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Genetic Transformation of *Azotobacter vinelandii*

by

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James Lawrence Doran

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Abstract

Various physiological aspects of competence development and DNA binding and uptake by *Azotobacter vinelandii* OP strain UW1 were investigated.

Calcium was required in the competence induction medium of strain UW1 for the maintenance of membrane integrity and for the development of high levels of transformation competence. The detrimental effect on competence of growth in calcium-limited medium was partially reversible by exposing cells to calcium or strontium, but not magnesium, in the presence of a 60,000 molecular weight (60K) glycoprotein.

This 60K glycoprotein was demonstrated to be the primary, if not sole, component of a regularly ordered layer located external to the outer membrane. The interactions between glycoprotein molecules required to form a regular lattice were apparently promoted by calcium ions but not magnesium ions. Although an organized surface layer seemed to be a requirement for high levels of competence, neither the presence of a regular array nor the 60K glycoprotein was necessary for normal levels of DNase-resistant binding of transforming DNA.

Competent and noncompetent strain UW1 bound equivalent amounts of DNA in a DNase-resistant state. Competent strain UW1 participated in two forms of DNase-resistant DNA binding to the cell envelope. DNA binding to "type 1" receptors occurred in the absence of genetic transformation and was typified by the indiscriminant binding of either homologous or heterologous (nontransforming) DNA. DNA receptors of "type 2" are proposed to be specifically responsible for DNA binding prior to uptake and may be responsible for the discrimination against transformation by heterologous DNA.

DNase-resistant DNA binding demonstrated a brief lag of 1 to 3 sec and was saturated, in terms of transformation, 5 to 10 min after the initial exposure of cells to excess homologous DNA. Competent cells exposed to saturating concentrations of transforming DNA could not be transformed a second time in the same competence cycle.

Approximately 15 to 20 per cent of the DNA bound to cells in a DNase-resistant state was transported into the cytoplasm in a biologically active, apparently double-stranded, form. DNA uptake was a temperature-sensitive event. Evidence is presented that DNA uptake may occur at competence-specific, heat-labile sites of adhesion between the inner membrane and the cell wall.

Newly acquired donor DNA markers were expressed in recipient cells beginning 60 to 80 min after initial DNA binding.

Most components of the DNA binding and uptake system were not identified although several envelope proteins were possibly implicated in the process. The growth of cells in calcium-limited medium led to the release of several competence-dominant proteins and low levels of transformability. Distilled water washing of these cells released a competence-specific protein (pI 5.19) and prevented calcium- and glycoprotein-mediated competence recovery. Washing calcium-sufficient cells with distilled water or 0.2 M $MgSO_4$ diminished competence and extracted four common proteins. The observation that competence recovery required protein synthesis cannot be interpreted to implicate certain of these proteins in genetic transformation because of the

generally disruptive effects of these treatments on the cell envelope. Moreover, protein synthesis was observed to be a normal requirement for competence induction.

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I. General Introduction

The term "genetic transformation" refers to the process by which bacteria acquire new heritable traits through the incorporation of exogenous DNA. This process can be envisioned to occur in sequential stages. The first of these stages involves DNA binding to the cell surface. This is followed by DNA uptake across the cell envelope. The final stage, which is necessary for the expression of chromosomal markers, is the integration of transforming DNA into the host chromosome. Competence, for genetic transformation, describes that physiological state in which bacterial cells possess the ability to bind and transport transforming DNA. Competence defined as the ability of cells to bind DNA in a state such that it is insensitive to externally added deoxyribonuclease (DNase) (Lerman and Tolmach, 1957; Smith et al., 1981) is no longer applicable, as transformable and nontransformable *Azotobacter vinelandii* equally demonstrate this ability (this study). Competence is not a constitutive characteristic of most bacteria but can be induced under the appropriate conditions.

The phenomenon of genetic transformation was originally reported by Griffith (1928). This observation of the transformation of capsular types in pneumococcus by in vivo experimentation in mice was soon confirmed by Neufeld and Levinthal (1928). Later studies by Dawson and Sia (1931) demonstrated that this transformation would also occur in vitro. Investigations to determine the nature of the transforming principle culminated in a study by Avery et al. (1944) in which evidence was presented which suggested that DNA may be the conveyor of heritable traits. Although a series of investigations by Avery and coworkers utilized this transformation system to verify these findings (Austrian and MacLeod, 1949; McCarty and Avery, 1946a,b), it remained for Hershey and Chase (1953) to provide evidence leading to the general acceptance of DNA as the genetic material (Pollack, 1970).

Although the discovery of the nature of the genetic material highlights the significant role that genetic transformation has played in our understanding of biology, this phenomenon has continued to be important for developments in the fields of bacterial genetics and molecular biology. Most notably, genetic transformation has provided the method of choice for the fine mapping of genetic mutations in bacteria. More recently, the discovery of a method for the genetic transformation of *Escherichia coli* (Cohen et al., 1972; Mandel and Higa, 1970) provided one of the cornerstones for the development of "genetic engineering".

The phenomenon of genetic transformation has been described in many bacteria including member(s) of the *Rhodospirillaceae* (Tucker and Pemberton, 1980), *Spirillaceae* (Mishra et al., 1979), *Pseudomonadaceae* (Chakrabarty et al., 1975; Mercer and Loutit, 1979), *Azotobacteriaceae* (David et al., 1981; Page and Sadoff, 1976a), *Rhizobiaceae* (Kern, 1969; Raina and Modi, 1972), *Enterobacteriaceae* (Cohen et al., 1972; van Rensburg, 1971), *Neisseriaceae* (Juni and Janik, 1969; Sarubbi and Sparling, 1974), *Micrococcaceae* (Lindberg and Novick, 1973), and *Bacillaceae* (Coukoulis and Campbell, 1971; Gwinn and Thorne, 1964). Despite this, our current understanding of the process of genetic transformation has resulted from studies of relatively few organisms.

These include, principally, *Streptococcus pneumoniae*, *Streptococcus sanguis*, *Bacillus subtilis*, and *Haemophilus influenzae*, and, to a lesser extent, *Neisseria gonorrhoeae* and *E. coli*.

The process of genetic transformation of bacteria by chromosomal DNA has been proposed to occur in four stages (Lacks, 1977a). The initial stage involves DNA binding to the cell surface in such a manner that it remains sensitive to externally added DNase. This is followed by DNA attaining a DNase-resistant state. This step may occur concomitant with the third stage of the process, DNA transport into the cytoplasm. The final stage involves recombination between the transforming DNA and the host chromosome. This final stage of transformation is not of particular concern to the studies described in this thesis and, therefore, will not be dealt with in detail in this introduction. Several reviews of the literature concerned with genetic transformation have included a discussion of aspects of recombination and the reader is referred to them (Dubnau, 1976; Fox, 1978; Hotchkiss and Gabor, 1970; Notani and Setlow, 1974; Smith et al., 1981; Venema, 1979). As well, the current knowledge and hypotheses concerning the molecular events occurring during genetic recombination have been summarized in a recent review article by Dressler and Potter (1982).

The systems developed for the genetic transformation of bacteria can be divided into those in which the cells are naturally induced to competence under certain conditions of growth or metabolism and those in which competence is "artificially induced" in noncompetent cells.

The bacteria which are artificially induced to competence include *E. coli* (Cohen et al., 1972), *Salmonella typhimurium* (Lederberg and

Cohen, 1974), *Pseudomonas putida* (Chakrabarty et al., 1975), *Pseudomonas aeruginosa* (Sano and Kageyama, 1977), and *Staphylococcus aureus* (Lindberg et al., 1972). Competence is induced in these cells primarily by treatment with high concentrations of $CaCl_2$ or $MgCl_2$ and various temperature treatments. This regime renders the majority of the population nonviable. The frequency of genetic transformation of the remainder of the population by a single marker is typically quite low (10^{-4} to 10^{-8} transformants per viable cell). Despite the low viability and poor transformability, these transformation systems offer the principal advantage, in terms of the cloning of recombinant DNA or the creation of heterogenotes, that the transforming DNA may be homologous or heterologous with respect to the transformation recipient (Brown et al., 1981). The fact that the genetics of *E. coli* is relatively well understood favours the use of this organism for these purposes. The little that is known about DNA binding and uptake in these transformation systems has been gained from studies of *E. coli*. This information will be discussed following a discussion of the early stages of genetic transformation of bacteria which are naturally induced to competence.

Within the group of bacteria which are naturally induced to competence, important distinctions can be made between DNA uptake by Gram-positive and Gram-negative organisms. This has led to the proposal of somewhat different mechanisms for DNA binding and transport in each case (Smith et al., 1981).

The Gram-positive bacterial transformation systems of *Streptococcus pneumoniae* and *Bacillus subtilis* are certainly the most well-characterized and excellent reviews of each are available. The earlier investigations have been reviewed by Tomasz (1969). A more recent review article by Lacks (1977a) emphasizes DNA binding and uptake by *S. pneumoniae* while the genetic transformation of *B. subtilis* is discussed in an article by Dubnau (1976). Comprehensive reviews of all the well-studied transformation systems have been written by Venema (1979) and Smith et al. (1981).

Streptococcus pneumoniae becomes competent for genetic transformation through exogenous activation by an autocatalytic peptide, referred to as a "competence factor", which becomes sufficiently active when cultures attain a critical cell density (Leonard and Cole, 1972; Tomasz, 1966, 1970; Tomasz and Hotchkiss, 1964). It has been postulated that the initiation of competence induction involves an interaction between the competence factor and a specific cell surface receptor present on noncompetent cells (Horne et al., 1977; Lacks, 1977a) which may lead to the observed changes in the level of expression of various genes during competence induction (Morrison and Baker, 1979). A role for a competence factor in the *S. sanguis* transformation system has also been reported (Ceglowski and Dobrzanski, 1974; Dobrzanski et al., 1970; Pakula et al., 1970; Pakula et al., 1971; Perry, 1974).

Although a competence factor has been tentatively identified in *Bacillus subtilis* (Akrigg and Ayad, 1970; Akrigg et al., 1967) which may have autolytic activity, there is also evidence that competence development may be solely internally regulated (Goldsmith et al., 1970). *B. subtilis* becomes competent in early stationary phase or after a shift to a nutritionally deficient medium (Spizizen et al., 1966). When optimally competent, cells of *B. subtilis* are no longer dividing (Sadaie and Kada, 1983; Singh and Pitale, 1974). Unlike competent *S. pneumoniae* cultures, in which virtually all cells become competent (Tomasz and Hotchkiss, 1964), competent cultures of *B. subtilis* attain little more than 20 per cent competence (Hadden and Nester, 1968; Vermeulen and Venema, 1971). Competence induction in *B. subtilis*, *S. pneumoniae* and *S. sanguis* occurs concurrently with changes in the overall metabolic activities of the cell and is dependent upon protein synthesis (Dooley et al., 1971; Espinosa et al., 1980; Horne and Perry, 1974; Leonard, 1973; Morrison and Baker, 1979; Sadaie and Kada, 1983; Tomasz, 1970).

Sufficient progress has been made towards an understanding of the transformation process in competent *S. pneumoniae* and *B. subtilis* and sufficient similarity exists between these transformation systems and that of *S. sanguis* that a simplified general model for DNA binding and uptake by Gram-positive bacteria can be formulated.

Competent cells and transforming DNA contact one another by virtue of random collisions (Tomasz, 1969). The initial association between the cell and a molecule of DNA is a reversible form of DNA binding in which DNA is susceptible to digestion by externally added DNase and can be removed by shear forces (Dubnau and Cirigliano, 1972a; Seto et al., 1975). Membrane-bound DNA binding factors have been reported (Ceglowski et al., 1980; Seto et al., 1975; Smith et al., 1983) which may participate in this initial, possibly end-wise, DNA binding. DNA binding is an energy dependent process (Dubnau and Cirigliano, 1974; Lacks et al., 1974). In *S. pneumoniae* DNA binding is accompanied by the introduction into the DNA of single-stranded nicks spaced at approximately regular intervals about 6000 base pairs apart (Lacks, 1979; Lacks and Greenberg, 1976). The binding of DNA destined to transform cells (Morrison and Guild, 1973a) is specific for unmodified duplex DNA of homologous or heterologous origin (Ceglowski et al., 1975; Ciferii et al., 1970; Lacks, 1977b, Lerman and Tolmach, 1957; Rownd et al., 1968; Soltyk et al., 1975).

Prior to DNA uptake and preliminary to DNase-resistant DNA binding, double-stranded breaks are introduced into the DNA (Dubnau and Cirigliano, 1972b; Morrison and Guild, 1973a). Characteristic of Gram-positive transformation systems, the double-stranded DNA fragments are converted to a single strand of contiguous DNA, prior to or during uptake, with concurrent degradation of the opposite strand to acid-soluble products (Lacks, 1962; Lacks et al., 1967; Morrison and Guild, 1972, 1973b; Piechowska and Fox, 1971; Venema and Canosi, 1980). This linear uptake of DNA (Morrison and Guild, 1973b; Strauss, 1965, 1966) is an energy-dependent process (Seto and Tomasz, 1974; Strauss, 1970) and requires the presence of a membrane bound nuclease (Joenje et al., 1975; Joenje and Venema, 1975; Lacks and Greenberg, 1967; Lacks et al., 1974, 1975; Lacks and Neuberger, 1975; Mulder and Venema, 1982a,b). Magnesium ions are required for DNA uptake, possibly for the activity of this nuclease (Buitenwerf and Venema, 1977; Garcia et al., 1978; Lacks, 1977a,b; Lacks and Greenberg, 1973; Young and Spizizen, 1963).

It has been proposed that an exonuclease may act as a "DNA translocase" promoting the uptake of a single strand of DNA by a mechanism which is possibly facilitated by the degradation of the opposite strand

(Lacks, 1977a; Lacks et al., 1974). This model may not apply to either cells of *B. subtilis* or *S. sanguis* as some single-stranded DNA has been observed external to the cytoplasmic membrane of these cells (Smith et al., 1981; Strauss, 1970). The process of DNA uptake by *S. pneumoniae* also differs from that of *B. subtilis* in that it demonstrates a separate requirement for low concentrations (0.3-1.0 mM) of calcium ions which cannot be fulfilled by magnesium ions (Seto and Tomasz, 1976). DNA uptake by *B. subtilis* appears to demonstrate a unique requirement for proton motive force (van Nieuwenhoven et al., 1982). The DNA transport mechanisms of all Gram-positive bacteria do not discriminate against heterologous DNA (Lacks et al., 1977a; Lopez et al., 1980a,b; Soltyk et al., 1975).

DNA uptake is a saturable process (Anagnostopoulous and Spizizen, 1961; Fox and Hotchkiss, 1957). This implies that a limited number of DNA uptake sites exist on the surface of the competent cells. It has been estimated that competent *B. subtilis* and *S. pneumoniae* possess 30 to 60 sites for DNA transport (Fox and Hotchkiss, 1957; Lacks, 1977a; Singh, 1972). A detailed consideration of the kinetics of DNA binding and uptake has been presented by Lacks (1977a).

Other than the implication of certain nuclease activities in DNA uptake, the nature of the components of the cell envelope which form the DNA transport system remains to be determined. There is some evidence to suggest that transforming DNA bound by *B. subtilis* may be associated specifically with mesosomal membranes extruded at the growing regions of the cell and that these mesosomes may facilitate DNA uptake and integration (Erickson, 1970; Vermeulen and Venema, 1974a,b; Wolstenholme et al., 1966).

Immediately following DNA uptake by Gram-positive bacteria the DNA is said to be in an "eclipse-phase" or "biological eclipse" since no detectable donor DNA transforming (biological) activity can be recovered from transformed cells by virtue of the single-stranded nature of this DNA. In this eclipse-phase the DNA is associated with one or more competence related proteins which provide protection for the DNA from intracellular, or in the case of *B. subtilis* and *S. sanguis* possibly extracellular, nuclease digestion (Buietenwerf and Venema, 1977; Dubnau and Davidoff-Abelson, 1971; Eisenstadt et al., 1975; Morrison, 1977; Morrison and Baker, 1979; Pieniazek et al., 1977; Raina et al., 1979; Raina and Ravin, 1977). The transforming activity of the donor marker reappears following integration of this DNA into the host chromosome.

Gram-negative bacteria which are naturally induced to competence appear to have developed a fundamentally different mechanism for DNA binding and uptake from Gram-positive bacteria. The model emerging for this process is based primarily on studies of *H. influenzae*, however, the similarities between this transformation system and that of *Neisseria gonorrhoeae* suggest that this model may be generally applicable to Gram-negative bacteria.

Competence development in *H. influenzae* occurs when cells are shifted into a nongrowth medium which supports protein synthesis (Herriott et al., 1970). It is possible, by this means, to generate cultures in which essentially all cells are competent. Piliated strains of *N. gonorrhoeae*, but not unpiliated strains, are constitutively competent (Biswas et al., 1977; Sparling, 1966). No "competence factors", such as those reported in Gram-positive transformation systems, have been observed in Gram-negative transformation systems (Smith et al., 1981).

DNA uptake by transformable, metabolically-active, *H. influenzae* cells requires 1 mM calcium and 100 mM sodium or potassium (Barnhart and Herriott, 1963; Smith et al., 1981). The process is proposed to occur in three stages (Deich and Smith, 1980) generally similar to those proposed by Lacks (1977a) for *S. pneumoniae*. The first step is a reversible (exchangeable) binding of DNA to the cell surface. This is followed by an irreversible adsorption of DNA into a DNase-resistant state and, subsequently, DNA transport across the cell envelope.

Unlike the Gram-positive transformation systems, heterologous DNA will not serve to transform these Gram-negative bacteria. Although heterologous DNA is capable of binding to the cell surface of *H. influenzae* cells, it is not bound in a DNase-resistant state and is unable to block homologous DNA uptake (Scocca et al., 1974; Smith et al., 1981). Cells of *N. gonorrhoeae* demonstrate similar discrimination against heterologous DNA (Dougherty et al., 1979). Discrimination by *H. influenzae* cells could not be attributed to recognition of the specific modification pattern present on the transforming DNA (Chung and Goodgal, 1979) or to the digestion of heterologous DNA by recipient cell restriction enzymes (Stuy, 1976). Rather, the basis for discrimination resides solely in the recognition of a specific 11 base pair DNA sequence which is more common in homologous DNA than heterologous DNA (Danner et al., 1980; Danner et al., 1982; Sisco and Smith, 1979).

It has been estimated that there are approximately 600 so-called "uptake sites" in the genome of *H. influenzae* (Sisco and Smith, 1979). Sequence-specific DNA uptake by *N. gonorrhoeae* cells has also been reported (Graves et al., 1982; Mathis and Scocca, 1982).

Under normal conditions, DNA uptake by competent cells of *H. influenzae* and *N. gonorrhoeae* is specific for duplex DNA. The DNA remains in a double-stranded form during uptake, a characteristic typical of Gram-negative transformation systems (Barnhart and Herriott, 1963; Biswas and Sparling, 1981; Mulder and Doty, 1968; Voll and Goodgal, 1961). *H. influenzae* cells release little acid-soluble material resulting from the digestion of transforming DNA (Stuy and van der Have, 1971) and there is no "eclipse-phase" in recoverable transforming activity following DNA uptake (Stuy, 1965; Voll and Goodgal, 1961). There is some evidence, however, that single-stranded regions are formed in the transforming DNA prior to integration (LeClerc and Setlow, 1975). Competent *H. influenzae* cells exposed to saturating concentrations of homologous DNA irreversibly bind DNA in a DNase-resistant state in 1 to 3 sec and DNA uptake may be complete after 1 minute (Deich and Smith, 1980; Smith et al., 1981; Stuy and Stern, 1964). This DNA appears to be integrated into the host chromosome sometime between 10 and 60 min after adsorbing to the cell surface (Stuy, 1965).

DNA binding to both *H. influenzae* and *N. gonorrhoeae* cells is saturable (Deich and Smith, 1980; Dougherty et al., 1979; Stuy and Stern, 1964). It has been proposed that a limited number of DNA receptors exist on the surface of the competent cells and that each is available to bind only one molecule of DNA. It is estimated that *H. influenzae* possess 4 to 8 DNA receptors per competent cell (Deich and Smith, 1980). These receptors are presumably responsible for the recognition of the specific uptake sites on the DNA.

DNA binding receptors on competent cells of *H. influenzae* are located on competence-specific membrane "blebs" which extend from the cell surface (Concino and Goodgal, 1982; Deich and Hoyer, 1982; Kahn et al., 1982). DNA may be bound in a DNase-resistant state to preparations of isolated vesicles and the characteristics of this binding were the same as for binding to intact, competent cells (Deich and Hoyer, 1982). Competent *H. parainfluenzae* cells contain similar membrane blebs which appear to be located intracellularly following the uptake of transforming DNA. Although the membrane blebs on cells of *H.* were not similarly incorporated, they were observed to be located adjacent to sites of adhesion between the inner and outer membrane (Bayer, 1979) which may play some role in DNA uptake (Kahn et al., 1982).

The protein composition of the competence-specific membrane blebs from *H. influenzae* cells was determined to be similar to that of the outer membrane fraction (Kahn et al., 1982). Several attempts have been made to identify the surface DNA receptors. The observation that antibodies directed against competent cells block DNA uptake by competent cells, while antibodies directed against noncompetent cells do not, suggests the existence of a competence-specific DNA receptor (Bingham and Barnhart, 1973). Although the synthesis of several envelope polypeptides, many of which are characteristic of competent cells, has been correlated with the development of competence and the ability of cells to bind and transport DNA (Concino and Goodgal, 1981; Zoon et al., 1976; Zoon and Scocca, 1975), none of them have been demonstrated to have a direct role in transformation. One, or possibly two, membrane proteins of apparent molecular weights 22,500 (Kooistra et al., 1980) and 29,000 (Concino and Goodgal, 1982) have been implicated in DNA uptake as has an, apparently periplasmic, DNA-binding protein (Sutrina and Scocca, 1979). The certain identity of the components of the DNA binding and uptake system of *H. influenzae* remains to be determined.

The genetic transformation of bacteria which are artificially induced to competence by a variation of the method of Cohen et al. (1972) probably represents the third fundamentally distinct mechanism for DNA binding and uptake. The genetic transformation of *E. coli* has served as the model system in which to study this process.

A technique for the preparation of *E. coli* competent for transfection (transformation by bacteriophage DNA) by treating cells with $CaCl_2$ was developed by Mandel and Higa (1970). This technique was successfully utilized by Cohen et al. (1972) for the preparation of *coli* competent for transformation.

The binding of DNA to $CaCl_2$-treated *E. coli* is promoted by any of several divalent cations, including calcium and magnesium. Apparently most cells in the population participate in this form of DNase-sensitive DNA binding although very few cells are subsequently transformed (Weston et al., 1981). The cells were not capable of binding this DNA in a DNase-resistant state or becoming transformed until the population was briefly heated at 42°C (Bergmans et al., 1981; Cohen et al., 1972; Sabelnikov et al., 1975; Weston et al., 1981). DNase-resistant DNA binding and subsequent transformation absolutely requires the presence of calcium ions (Bergmans et al., 1981; Cosloy and Oishi, 1973a; Weston et al., 1981). The bulk of the DNA bound to cells was not taken up but remained bound to the outer membrane (Sabelnikov et al., 1975; Weston et al., 1981). That DNA which was taken up by competent cells was transported in a double-stranded form (Strike et al., 1979) by an apparently energy-independent transport mechanism (Sabelnikov and Domaradsky, 1981). This DNA uptake mechanism did not discriminate against heterologous DNA (Brown et al., 1981).

Unlike bacteria which are naturally induced to competence, it is uncertain whether DNA uptake by artificially-induced competent cells of *E. coli* involves an elaborate DNA transport system. Several investigators have proposed that competence development by *E. coli* may depend upon a loss of integrity of the membranes of the cell envelope (Bergmans et al., 1981; Cosloy and Oishi, 1973a; Weston et al., 1981). Indicative of this perturbation in the cell membranes is the release of substantial amounts of various periplasmic proteins concurrent with competence development (Bergmans et al., 1981) and the greater permeability of competent cells to certain antibiotics (Sabelnikov and Domarkadsky, 1981). Moreover, the treatment of lam B^- mutants of *E. coli* with 25 mM calcium has been demonstrated to increase the permeability of the outer membrane sufficiently to permit the passage of maltose and lysozyme (Brass et al., 1981). Consistent with the notion of artificially-induced regions of greater permeability in the outer membrane is the report that calcium-treatment of cells results in the generation of outer membrane particles of lipopolysaccaride and protein which are proposed to function as aqueous pores (van Alphen et al., 1979). Although this does not solve the problem of DNA transport across the inner membrane, these pores may transiently exist at adhesion sites between the inner and outer membranes (Bayer, 1979) or, alternatively, the high concentrations of calcium, which are known to promote membrane fusion (Poste and Allison, 1973; Verkeij et al., 1979), may artificially induce adhesion sites to form.

Certain characteristics of the process of DNA uptake by calcium-treated *E. coli* cells are more consistent with a chemically induced permeation of DNA through the cell envelope than exposure or induction of an efficient DNA transport mechanism. These include, the limited amount of DNA taken up by competent cells (Weston et al., 1981), the lack of discrimination against heterologous DNA typical of other Gram-negative bacteria and the lack of an energy-requirement for DNA transport. Furthermore, the extreme detrimental effect of the competence regime on cell viability (Cosloy and Oishi, 1973b) is not unexpected for a treatment which can generate cells permeable to such a large hydrophilic molecule as DNA. Therefore, the nature of DNA uptake by cells artificially induced to competence by treatment with $CaCl_2$ may have little relevance to the process of DNA uptake in other transformation systems.

Azotobacter vinelandii may be induced to competence either by an artificial means similar to *E. coli* (David et al., 1981) or by natural induction.

A. vinelandii is a relatively large, obligately aerobic, Gram-negative bacteria which is notable for its ability to fix atmospheric nitrogen (N_2) and for its ability to form cysts (Becking, 1974; Sadoff et al., 1975). A method for the natural induction of competence in *A. vinelandii* was originally reported by Page and Sadoff (1976a). This technique for competence development and transformation involved mixing cells with a crude lysate preparation of DNA (Juni, 1972) on solid medium. The cells grew to competence and became transformed during a subsequent 24 h incubation period. Although this method proved to be a very effective means of transformation (Maier et al., 1978; Page, 1978; Page and Sadoff, 1976b), it did not readily lend itself to examination of those physiological events occurring during competence development or to an elucidation of the conditions necessary for DNA binding and uptake. The two developments which solved these problems were the discovery of a means for competence induction in liquid culture (Page and von Tigerstrom, 1978) and the development of a liquid transformation assay system (Page and von Tigerstrom, 1979).

Iron-limitation or molybdenum-limitation is essential for the induction of competence in *A. vinelandii* (Page and von Tigerstrom, 1978, 1982). Competence development appears to be initiated as the cells reach a state of oxygen-limitation (Page, 1982). Competence development in iron-limited Burk medium requires an appropriate source of carbon (glucose, sucrose, glycerol or mannitol) and optimal levels of competence are attained when a source of fixed nitrogen, which is a repressor of nitrogenase, is also present (Page, 1982; Page and von Tigerstrom, 1978). Competence can be induced in a population of nitrogen-fixing cells, however, provided the medium is buffered to maintain a neutral or slightly alkaline pH (Page, 1982).

Competent cells appear in a population of *A. vinelandii* following 9 to 12 h incubation in iron-limited Burk medium containing glucose and ammonium acetate (Page and von Tigerstrom, 1978). These cells appear to be in the late exponential or early stationary phase of growth. Cells in a competent population are smaller and more pleomorphic than noncompetent cells prepared by growth in Burk medium (Page, 1982; Page and von Tigerstrom, 1978). This pleomorphism and, perhaps, competence development itself may be related to the presence of an autolysin which is active over the pH range required for competence development and which appears to be more active in competent than noncompetent cells (Page, 1982). Concurrent with oxygen limitation and the development of competence is the intracellular deposition of large amounts of poly-β-hydroxybutyrate. Consideration of this and the inhibitory effects of increased respiratory and/or nitrogenase activity on competence suggests that the attainment of optimal levels of transformability is dependent upon the cell maintaining a high ratio of NAD(P)H to NAD(P) (Page, 1982). A population of cells will remain highly competent for approximately 10 to 12 h after which extended incubation results in a rapid decline in competence (Page and von Tigerstrom, 1978).

Some of the conditions necessary for the transformation of competent *A. vinelandii* have been determined (Page and von Tigerstrom, 1979). The transformation of cells in buffer demonstrates an optimal pH range of 6.0 to 8.0 and absolutely requires magnesium ions. No other metal ions were required for transformation. The process of transformation was sensitive to temperature demonstrating an optimum at 30°C. A dramatic loss of transformability was observed in cells transformed, or pretreated, at or above 37°C.

Cells transformed to Nif$^+$* or to antibiotic resistance required 15 to 20 h incubation for phenotypic frequency stabilization. This may have reflected a lag in cell division and hence a delay in nuclear segregation. Cells transformed to antibiotic resistance required prior incubation on nonselective medium to allow phenotypic expression before plating on selective medium (Page and von Tigerstrom, 1979).

While studies of *Azotobacter* transformation are possibly important to a larger understanding of DNA binding and uptake in Gram-negative bacteria, characterization of the *Azotobacter* transformation system may have practical application. *A. vinelandii* is an attractive alternative to *E. coli* as bacterial host for recombinant DNA vectors. Unlike *E. coli*, *A. vinelandii* has no known plant or animal host and is not known to be pathogenic. *A. vinelandii* is routinely transformed at frequencies three or four orders of magnitude greater than *E. coli* (Cohen et al., 1972; Page and von Tigerstrom, 1978). As well, it is much less expensive to culture and maintain *A. vinelandii* than *E. coli*. Considering the recent interest shown in the genetic engineering of nitrogen-fixing bacteria for agricultural purposes, the *Azotobacter* transformation system has the potential of providing a very valuable tool.

*Nif$^+$ designates a nitrogen-fixing phenotype.

II. Materials and Methods

Detailed descriptions of experimental procedures used in more than one study, and those descriptions which do not appear elsewhere are presented in this chapter. Descriptions of pertinent procedures appear in specific detail in the individual chapters.

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. *Azotobacter vinelandii* OP strain UW1 (Nif^- $capsule^-$ rifampicin-sensitive streptomycin-sensitive) was used almost exclusively as the transformation recipient. *A. vinelandii* OP strain UW91 (Nif^-) was used as a transformation recipient on a single occasion. Both recipient strains were derived by N-methyl-N-nitrosoguanidine induced mutagenesis of *A. vinelandii* OP strain UW (Fisher and Brill, 1969). Strain UW1 possessed mutation(s) in a gene involved in the regulation of nitrogenase expression (Bishop et al., 1977, Kennedy and Robson, 1983). This gene appeared to be similar in function to the *nif A* gene of *Klebsiella pneumoniae* (Buchanan-Wollaston et al., 1981). Strain UW91 produces an inactive component II (Fe-component) of the nitrogenase complex (Bishop and Brill, 1977).

All diazotrophs listed in Table 1 were used as sources of transforming DNA. *Clostridium pasteurianum* was grown anaerobically at 37°C on modified Burk medium (Table 2) containing 2% sucrose and 2 µg biotin/liter. *K. pneumoniae* was maintained at 37°C on nutrient agar

TABLE 1. Bacterial Strains.

Organism	Strain	Relevant Phenotype[a]	Source
Azotobacter vinelandii OP	UW	Nif^+ $Capsule^-$	W. Brill, University of Wisconsin
A. vinelandii OP	UW1	Nif^- $Capsule^-$	W. Brill, University of Wisconsin
A. vinelandii OP	UW91	Nif^- $Capsule^-$	W. Brill, University of Wisconsin
A. vinelandii ATCC 12837	113	Nif^+ $Capsule^+$ Rif^r	
A. vinelandii ATCC 12837	114	Nif^+ $Capsule^+$ Str^r	
A. chroococcum ATCC 7486		Nif^+	
A. paspali ATCC 23368		Nif^+	
A. beijerinckii ATCC 19360		Nif^+	
Azomonas macrocytogenes ATCC 12335		Nif^+	
A. insignis		Nif^+	H. Sadoff, Michigan State University
Beijerinckii indica ATCC 19360		Nif^+	
Rhizobium meliloti	17A	Nif^+	J. Tiedje, Michigan State University
R. trifolii	CC10	Nif^+	W. Page, University of Alberta
Klebsiella pneumoniae ATCC 13883		Nif^+	
Clostridium pasterianum NCIB 9486		Nif^+	
Escherichia coli HB 101			
E. coli J53			D. Taylor, University of Alberta

[a] Abbreviations: Nif, N_2-fixing; Rif^r, rifampicin-resistant; Str^r, streptomycin-resistant.

TABLE 2. Composition of Burk Medium[a]

K_2HPO_4	0.8 g	5mM phosphate buffer, pH 7.1
KH_2PO_4	0.2 g	
$MgSO_4 \cdot 7H_2O$	0.2 g	0.81 mM
$CaSO_4 \cdot 2H_2O$	0.08 g	0.58 mM
$FeSO_4 \cdot 7H_2O$[b]	5.0 mg	18 µM
$NaMoO_4$	2.5 mg	1.0 µM
CH_3COONH_4	1.1 g	14 mM
D-Glucose	10 g	1 %
Distilled H_2O	to 1000 ml	
Agar (if required)	18 g	1.8 %

[a] After Page and Sadoff, 1976a.

[b] Iron-limited Burk medium (competence induction medium) was Burk medium without added $FeSO_4 \cdot 7H_2O$.

(Difco Laboratories, Detroit, Mich.) containing 1% glucose. strains were grown at 30°C in yeast extract (Difco) agar containing 1% glucose. *Azotobacter paspali* and *Azotobacter beijerinckii* were propagated at 30°C on nitrogen-free Burk medium containing 1% sucrose as the carbon source. All other Nif$^+$ strains were grown at 30°C on nitrogen-free Burk medium. Nif$^-$ strains were grown on Burk medium at 30°C. Liquid cultures of *A. vinelandii* required proper aeration and were shaken at 170 rpm in a water bath gyrotory shaker model G-76 (New Brunswick Scientific Co., New Brunswick, N.J.). Burk buffer, pH 7.2, was Burk medium without glucose or ammonium acetate.

Escherichia coli strains used as hosts for various plasmids (Table 3) were grown at 37°C in Brain Heart Infusion broth (Difco) containing 10 to 20 µg/ml of an antibiotic appropriate to promote the retention of the pertinent plasmid.

Preparation of transforming DNA

Crude lysate DNA preparations were formed by resuspending cells from slant cultures to approximately 5 x 10^9 cells/ml in 15 mM saline-15 mM sodium citrate buffer, pH 7.0, (SSC) containing 0.05% sodium dodecyl sulfate (SDS) and heating at 60°C for 60 min (Page and Sadoff, 1976a). Suspensions of strains other than those of *A vinelandii* were heated at 60°C for up to 6 h to destroy all viable cells.

For some experiments the DNA was partially purified by extracting the crude lysates twice with 0.75 volume of liquified phenol. The deproteinized sample was treated with RNase A (final concentration, 100 μg/ml) for 30 min at 37°C. The DNA was then purified free of most contaminating oligoribonucleotides and capsular material as described by Marmur (1961).

Purified samples of [^{32}P]-labeled DNA and nonradioactive DNA were prepared from strain UW to avoid the problems of removing large amounts of capsular material from the DNA lysate. Radiolabeling of DNA was carried out by the addition of 2.5 mCi of [^{32}P]orthophosphate to 400 ml of modified Burk medium containing 1 mM potassium phosphate buffer, pH 7.1, and 1 x 10^7 cells/ml and incubating the culture at 30°C for 48 h. The cells were then pelleted from the culture and washed twice by centrifugation with 100 ml of Burk buffer to remove extraneous [^{32}P]-labeled material. The resulting cell pellet contained approximately 55% of the radioactivity present in the culture. The cells were lysed by suspending the cell pellet in 100 ml of SSC-SDS and heating the mixture for 2 h at 60°C. The crude lysate was cooled and treated with proteinase K (final concentration, 100 μg/ml). The [^{32}P]DNA was further purified as described above and then separated from residual contaminating [^{32}P]oligoribonucleotides by electrophoresis in 20 mM Tris (hydroxymethyl) aminomethane (Tris)-2 mM ethylenediaminetetracetic acid (EDTA)-50 mM sodium acetate buffer (pH 7.8)-0.5% agarose gels at 50 V for 4.5 h. The position of the band of [^{32}P]DNA was determined by autoradiography using Kodak XOMAT X-ray film. This portion of the gel was excised and the agarose was dissolved in KI as described (Smith, 1980). [^{32}P]DNA was bound to hydroxylapatite (HAP) (BioGel HTP, BioRad Laboratories). The HAP-[^{32}P]DNA was washed free of agarose and KI by a modification of the method of Smith (1980). The HAP-[^{32}P]DNA was not formed into a column as better recoveries of [^{32}P]DNA were obtained by batch treatment in an Erlenmeyer flask. Twenty to thirty percent of the [^{32}P]DNA was eluted in 1 M sodium phosphate-1 mM EDTA, pH 6.7. The solution was dialyzed twice in 48 h against 4 L of SSC at 4°C to reduce the concentration of sodium, phosphate, EDTA and residual KI. Samples were concentrated by dialysis against polyethylene glycol (PEG 20,000). The final preparation of DNA was biologically active in genetic transformation. The specific activity of the preparations varied between batches and with the age of preparation. Nonradioactive DNA was prepared using a similar method.

DNA concentrations were determined by the Burton (1956) assay as modified by Giles and Meyers (1965) using calf thymus DNA as a standard.

The various plasmids used as prospective sources of transforming DNA are listed in Table 3. The common plasmid cloning vectors pBR 322, (Bolivar et al., 1977), its raised copy derivative pAT 153 (Twigg and Sherratt, 1980) and pACYC 184 (Chang and Cohen, 1978) were prepared by Dr. K.L. Roy, Department of Microbiology, University of Alberta, as was the plasmid pSA30. pSA30, a derivative of pACYC 184, contained an insertion of *K. pneumoniae* DNA including *nif* KDH and a portion of *nif* E (Cannon et al., 1979). The α and β subunits of component I of *Klebsiella* nitrogenase were encoded by *nif D* and *K*. The *nif H* gene coded for the structural protein of nitrogenase component II (Roberts et al., 1978). This region of *Klebsiella* DNA was homologous to *Azotobacter vinelandii* DNA (Ruvkin and Ausubel, 1980) as expected

TABLE 3. Plasmids.

Plasmid[a]	Relevant Genotype[b]	Source or Reference
pBR 322	tet^r	Bolivar et al (1977)
pAT 153	tet^r	Twigg and Sherratt (1980)
pACYC 184	cam^r	Chang and Cohen (1978)
pSA 30	tet^r *nif*KDH	Cannon et al (1979)
1010	str^r	D. Taylor, University of Alberta
J53-R1-307	tet^r kan^r	
J53-R1	cab^r	
pDT 833	tet^r	
pDT 831	tet^r kan^r	

[a] The host strain for all plasmids except pDT 833 and pDT 831 was *E. coli* HB101, pDT 833 and pDT 831 were maintained in *E. coli* J53.

[b] Abbreviations: tet^r, tetracycline resistance; *cam*, chloramphenicol; *str*, streptomycin; *kan*, kanamycin; *cab*, carbenicillin; *nif*, nitrogen fixation.

considering the complementarity of the nitrogenase components (Emerich and Burris, 1978). These plasmids were maintained in *E. coli* HB101 (Bolivar and Backman, 1979). Purified plasmid DNA was suspended in 10 mM Tris-1 mM EDTA buffer, pH 7.8. The plasmids R1, R1 307 and 1010 and the large plasmids pDT 833 and pDT 831 were purified from *E. coli* HB101 and *E. coli* J53 (Table 3) by the method of Crosa and Falkow (1981). The plasmid DNA preparations were examined by electrophoresis of the samples in 0.5% agarose-TEA gels for 8 h at 25 V and observation of nucleic acid material revealed by ethidium bromide staining (Sharp et al., 1973).

Purified bacteriophage ØW-14 DNA (Kropinski et al., 1973) in 1 mM EDTA-10 mM Tris buffer, pH 7.8, was provided by R.A.J. Warren (Department of Microbiology, University of British Columbia, Vancouver). When necessary, ØW-14 DNA was diluted in Burk buffer prior to use as a competing DNA. Salmon sperm DNA was dissolved in Burk buffer and was also used as a potential source of competing DNA.

Competence induction

Cells of *A. vinelandii* strain UW1 or strain UW91 were washed from 24 h slant cultures on Burk medium using iron-limited Burk medium (Table 1). The cell suspension was used to inoculate iron-limited Burk medium (Erlenmeyer flask - 40% culture volume/flask volume) to an initial optical density, measured at 620 nm, of 0.05-0.07. Competent cells were generated after 18 to 24 h incubation at 30°C and 170 rpm.

Transformation

Competent cells were transformed in Burk buffer, pH 7.2, containing 8 mM $MgSO_4$ (transformation assay buffer [Page and von Tigerstrom, 1978] using approximately 10 μg of DNA/10^8 viable cells. This concentration of DNA was approximately 10 fold greater than the concentration required to saturate the competent cells. In some experiments 20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2, was substituted for Burk buffer. DNase-resistant DNA binding was terminated after 20 min at 30°C by the addition of DNase 1 (final concentration 4 μg/ml). Nif^+ transformants were selected by plating on nitrogen-free Burk medium. Transformants which had acquired antibiotic resistance characteristics required a period of incubation prior to plating on selective medium (Page and von Tigerstrom, 1979). These transformants were detected on Burk medium containing 20 μg/ml rifampicin, 50 μg/ml chloramphenicol, 15 μg/ml tetracycline, 20 μg/ml streptomycin or 30 μg/ml carbenicillin.

Electrophoresis

a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Proteins were solubilized by boiling for 5 min or by freezing and thawing five times in sample buffer which contained, at final concentration, 0.063 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue. The samples were added to wells in 1.5 mm thick acrylamide gels formed in a Hoeffer model 220 or 221 electrophoresis cell. Electrophoresis at 8-10 mA through the 5% acrylamide stacking gel was followed by electrophoresis through the 9 or 10% acrylamide separating gel at 12 to 14 mA until the bromophenol blue dye front approached the bottom of the gel. The gels were stained in 0.1% Coomassie blue in isopropanol and acetic acid according to Fairbanks et al. (1971).

b) Isoelectric focusing

Isoelectric focusing (IEF) in 2 mm diameter tube gels was done according to the procedure of O'Farrell (1975). The gel mixture contained 9.2 M urea, 2% Triton X-100, 4% acrylamide, 0.2% N,N'-methylene bisacrylamide, 0.9% 5-7 ampholytes and 1.1% 3.5-10 ampholytes. IEF slab gels, 1.5 mm thick, were formed in a Hoeffer model 220 electrophoresis cell using the same gel mixture lacking Triton X-100 (Page and Doran, 1981). Isoelectric focusing at 400 V was carried out for 13 to 24 h. The gels were stained by the method of Fairbanks et al. (1971) except that the Coomassie blue R250 concentrations were reduced by at least 60% to avoid problems with background staining. The pH gradient across the gel was determined from 5 mm cross sections which were mascerated and soaked in double-distilled deionized water for 2 to 3 h at room temperature. The pH of each sample was measured using a Fisher model 230 pH meter.

Membrane isolation

Cells were disrupted by passage through a French pressure cell. Granules of poly-β-hydroxybutyrate were collected from the lysate by centrifugation (5000 rpm, 20 min). Membrane fragments were then isolated on sucrose gradients as described by Page and von Tigerstrom (1982). Fractions collected from the bottom of the gradients were assayed for absorbance at 280 nm, sucrose concentration and NADH oxidase activity (Osborn et al., 1972). The pooled peak fractions were also assayed for lipopolysaccharide (Osborn et al., 1972), protein (Lowry et al., 1951) and poly-β-hydroxybutyrate (Law and Slepecky, 1961) concentrations.

[^{14}C]-labeling of 42°C-treated cells during competence recovery

Iron-limited Burk medium containing 1% or 0.1% glucose was inoculated with strain UW1 to an initial optical density, measured at 620 nm, of 0.05. The culture which originally contained 0.1% glucose was growth limited, had a turbidity (620 nm) of 0.30 after 21 h incubation. This culture was noncompetent. Sterile culture supernatant was prepared from this culture by centrifugation (10,000 rpm, 10 min) and filtration (0.45-μm Millipore filter). The competent culture (1% glucose) which had attained an optical density (620 nm) of 1.5 following 21 h incubation was heated at 42°C for 25 min to destroy competence. These cells recovered competence upon further incubation at 30°C. Although competence recovery was greater if cells were incubated in original, or unheated, culture supernatant rather than fresh iron-limited Burk medium, original culture supernatant was not an ideal medium for labeling cells using [^{14}C]glucose as it was not carbon-limited (Page and von Tigerstrom, 1978). Therefore, 42°C-treated formerly-competent cells were resuspended in glucose-limited, culture supernatant fluid containing a 0.2% glucose supplement which allowed normal competence recovery. The culture was incubated for 1 h prior to the addition of 0.01% [^{14}C]glucose (50 μCi) supplement. Following 4 h further incubation [^{14}C]-labeled cells were harvested by centrifugation and membrane fractions were isolated and analyzed by SDS-PAGE. Protein bands were stained with Coomassie blue R250 as described by Fairbanks et al. (1971). Polysaccharide containing material was stained using a modified periodic acid-Schiff reagent stain (Page and Stock, 1974). Lipopolysaccharide was detected using a modified silver stain (Tsai and Frasch, 1982). [^{14}C]-labeled materials were detected by gel fluorography (Laskey, 1980) using Kodak XOMAT film exposed for 8 weeks at -70°C. Poly-β-hydroxybutyrate was eluted from the gel by soaking macerated gel slices in 0.5 ml of distilled water for 48 h at 4°C and its presence was confirmed by the method of Law and Slepecky (1961).

Analysis of fatty acids

Fatty acid methyl esters were prepared from membrane fractions isolated from sucrose gradients. The procedure used was supplied by Supelco Inc. (bulletin no. 767). The membrane fragments were harvested by centrifugation, resuspended in 5% NaOH-50% methanol and heated at 100°C for 30 min in sealed tubes to hydrolyze the phospholipids. The saponificate was cooled and the pH adjusted to 2.0 with 6N HCl. Fatty acid methyl esters were formed by the addition of 10%(w/v)BCl_3-CH_3OH (Supelco) and heating at 80°C for 5 min. The fatty acid methyl esters were then extracted into chloroform-hexane (1:4, v/v).

The methyl esters of fatty acids were identified by gas-liquid chromatography using a Tracor 560 gas chromatograph fitted with a glass column containing 3% SP-2100 DOH on 100-200 mesh Supelcoport. The retention times of the unknown fatty acid methyl esters were compared with those of known fatty acid methyl esters present in a standards mixture (Supelco). The relative amount of each fatty acid methyl ester was quantitated using a Hewlett-Packard 3370B integrator.

Freeze-etch electron microscopy

Cells to be used for freeze-etch electron microscopy were pelleted from freshly prepared cultures and washed once by centrifugation with one volume of 5 mM phosphate buffer, pH 7.1. The pellets were resuspended in the residual supernatant fluid remaining following decantation. A droplet of thick cell suspension on a 3 mm diameter gold disc was frozen in liquid Freon and stored in liquid nitrogen (Moor, 1969). Samples were usually freeze-etched the same day. The specimens were held at -100°C and were fractured and etched for 30 sec using a Balzers BA 360M apparatus (DeVoe et al., 1971). Replicas were formed by shadowing with platinum (20-25 Å) and carbon (200-250 Å). The organic material contaminating the replicas was digested by soaking the replicas in 70% sulfuric acid, washing in distilled water and then soaking in 5% hypochlorous acid. The replicas were mounted on 200 mesh Formvar coated copper grids. The replicas were observed in a Phillips 300 electron microscope at an accelerating voltage of 60 or 80 kV.

Measurement of nitrogenase activity

The acetylene reduction assay developed by Hardy et al. (1968) was used to determine the nitrogenase activity of liquid cultures. The assay was carried out using 2 ml of culture in a 10-ml Erlenmeyer flask sealed with a rubber serum stopper. The reaction was initiated by the injection of 1 ml of C_2H_2. The culture was incubated and at appropriate times samples of the gas phase were analyzed using a Tracor model 560 gas chromatograph fitted with Propak R (80-100 mesh) 2-m stainless steel column to determine the amount of C_2H_4 present (Page, 1982). The amount of C_2H_4 present was quantitated by electronic integration using a Hewlett-Packard model 3370B integrator. Nitrogenase specific activity was expressed as nanomoles of C_2H_4 produced/h/10^8 cells.

III. Recovery of Competence in Calcium-Limited *Azotobacter vinelandii*

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Summary

Azotobacter vinelandii cells required 0.5 mM calcium in the iron-limited competence induction medium. This requirement also was fulfilled by strontium, but not by magnesium. Cells pregrown in competence medium lacking calcium rapidly recovered competence with the addition of 0.5 mM calcium, provided they were suspended in the growth supernatant. A 60,000-dalton glycoprotein (pI 5.10) present in competent or noncompetent culture supernatants participated in calcium-mediated competence recovery. Cells grown in calcium-limited medium appeared to have leaky cell envelopes and released a diverse array of proteins into the culture supernatant and into distilled water washes of the cells, seven of which appeared to be more dominant in competent cells. Two distilled water washes of cells grown in calcium-limited medium did not prevent calcium-mediated recovery of competence in the culture supernatant. Four to six distilled water washes removed a competence-specific protein (pI 5.19) and prevented calcium-mediated recovery of competence in the culture supernatant.

Introduction

Azotobacter vinelandii cells become competent after 18 to 20 h of growth on iron-limited Burk medium (Page and von Tigerstrom, 1978). Competence development requires a suitable carbon source (glucose, sucrose, or mannitol) and a nitrogen source that is also a repressor of nitrogenase (ammonia, nitrate, or urea). Transformation of competent cells is achieved with crude lysate or purified DNA in buffer containing magnesium (Page and Sadoff, 1976; Page and von Tigerstrom, 1979). The requirement for magnesium appears to be absolute, but because the medium used for competence induction contains calcium, the involvement of this cation in the transformation system cannot be ruled out. Other well-studied transformation systems require calcium or magnesium, or both, for transformation, with little discrimination in the effectiveness of these cations (Lacks, 1977; Lacks and Greenberg, 1973). This report describes investigations of the requirement for calcium in the development of *Azotobacter* competence.

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Experimental Procedures

Bacterial Strains

A. vinelandii OP strain UW1 (capsule-Nif-), obtained from W. Brill (University of Wisconsin, Madison), was used as a transformation recipient. A nitrogen-fixing strain of *A. vinelandii* ATCC 12837 (strain 113) was used as a source of donor DNA. *Azotobacter* strains were maintained on Burk medium previously described (Page and Sadoff, 1976) containing 1.1 g of ammonium acetate per liter (BBGN). Burk buffer was Burk medium without added glucose and ammonium acetate. Liquid cultures were grown at 30°C in a water bath gyrotory shaker model G-76 (New Brunswick Scientific Co., New Brunswick, N.J.) at 176 rpm.

Transformation

Competent cells were formed after 18 to 20 h of growth in the iron-limited competence medium (OFe+N) previously described (Page and von Tigerstrom, 1978). Crude DNA from strain 113 was prepared by lysis in 15 mM saline-15 mM sodium citrate, pH 7.0, containing 0.05% sodium dodecyl sulfate (SDS), heated at 60°C for 60 min (Page and Sadoff, 1976). Competent strain UW1 cells were transformed in 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, containing 16 mM $MgSO_4$ (Page and von Tigerstrom, 1979). Transformation was terminated after 20 min at 30°C with the addition of DNase I. Nif^+ transformants were detected by plating on nitrogen-free Burk medium. A period of preincubation for expression of this phenotype was not required before plating on selective medium (Page and von Tigerstrom, 1979). Transformation frequency was calculated as the number of Nif^+ transformants detected per number of viable cells plated on selective medium. All determinations were repeated at least twice.

Washing Intact Cells

Competent cells were harvested after 20 h of growth, when they had reached a cell density of approximately 3 x 10^8/ml. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall model RC-5 centrifuge. The culture supernatant was pooled and sterilized by filtration (0.45-μm Millipore filter). The cell pellet was resuspended aseptically in 0.1 times the original volume, using distilled water and vigorous Vortex mixing. The cells were pelleted again (wash 1) by centrifugation for 7 min at 4°C. Successive washes were conducted similarly, using equal volumes of distilled water. The washes also were sterilized by filtration. The cell viability after each wash was estimated by a viable count corrected for cell loss by standardizing the turbidity of the cell suspensions.

The culture supernatant and distilled water washes were reduced in volume by freeze-drying. The dried culture supernatant was suspended in distilled water at 0.05 times the original volume. This was dialyzed against two 16-liter volumes of distilled water for 68 h at 20°C. After dialysis, the samples were lyophilized and then resuspended in a minimal volume of distilled water. The distilled-water washes were freeze-dried once and then resuspended without dialysis.

Samples for isoelectric focusing (IEF)-polyacrylamide gel electrophoresis (PAGE) were treated as reported above except that the cell pellet was first washed in Burk buffer to remove proteins which had been released in the culture supernatant and the fluorescent green pigment produced by iron-limited cells, and then the cells were washed with distilled water.

Isolation of Glycoprotein and Lipopolysaccharide

A glycoprotein of 60,000 daltons (60K) originally described by Schenk et al. (1977) was concentrated from pooled distilled water washes of competent OFe+N cells by precipitation with 20 mM $CaCl_2$ (final concentration). The white flocculent precipitate that formed after 20 min at 20°C was removed by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall model RC-5 centrifuge. The pellet was suspended in 40 mM Tris buffer, pH 7.8, with dropwise addition of 0.5 M EDTA, pH 7.0. The crude glycoprotein was dialyzed for 48 h at 20°C in Tris buffer to remove the EDTA. The protein content of the glycoprotein fraction was estimated by the method of Lowry et al. (1951).

LPS was prepared from strain UW1 grown for 24 h at 30°C in 4 liters of BBGN medium (cell density, 4 x 10^8/ml). LPS was extracted from the cells by the hot water-phenol method of Westphal and Jann (1965). The aqueous layer was dialyzed against distilled water for 3 days at 20°C and then precipitated with 0.025 M $MgCl_2$ for 24 h at 4°C. The precipitate was collected by centrifugation and dissolved in distilled water. LPS concentration was estimated after partial acid hydrolysis (Osborn, 1963) by the thiobarbituric acid assay, using 2-keto-3-deoxyoctonate as a standard (Weissbach and Hurwitz, 1959).

Concentration of Proteins Released into the Culture Supernatant

Strain UW1 was grown in 2.4-liter cultures to a cell density of approximately 10^8/ml, the level of competence was assayed, and the cells were removed by centrifugation at 15,000 rpm for 15 min at 4°C. The resultant culture supernatant was filter sterilized and concentrated to 600 ml, using an Amicon-DC2 hollow-fiber filter unit with a size cutoff of 10,000 daltons. The sample was lyophilized, suspended in 50 ml of distilled water, and dialyzed against 16 liters of distilled water for 48 h at 4°C. Lyophilization was repeated, and the sample was finally suspended in 2 ml of distilled water. This sample was passed through a column (2.5 by 40 cm) containing coarse Sephadex G-50 (Pharmacia, Uppsala, Sweden), using 40 mM Tris-hydrochloride, pH 6.8, as the eluant. This step removed the low-molecular-weight peptide-containing phenolic pigments from the other proteins. The nonpigmented fractions were pooled, lyophilized, suspended in 20 ml of distilled water, and dialyzed as before. The sample was concentrated by lyophilization to a final volume of 0.8 ml in distilled water. The protein concentration was determined by the method of Lowry et al. (1951).

Electrophoresis

SDS-PAGE was done according to the method of Laemmli (1970). Samples were added to an equal volume of sample buffer containing 0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.004% bromophenol blue and then boiled for 5 min before application to the gel (Laemmli, 1970). Electrophoresis was carried out in 1.5-mm-thick 9% acrylamide slab gels in a Bio-Rad model 520 electrophoresis cell for 16 h at 8 mA. The gels were stained in 0.1% Coomassie blue in isopropanol and acetic acid according to Fairbanks et al. (1971). The molecular weight of the glycoprotein was estimated by comparison of its relative mobility with that of standard proteins (Weber and Osborn, 1969). The glycoprotein nature of this band was confirmed by staining with a modified periodic acid-Schiff reagent (Page and Stock, 1974).

IEF-PAGE in vertical slab gels was a modification of the procedure described by O'Farrell (1975) for tube gels. Focusing was carried out in a Bio-Rad model 220 electrophoresis cell which formed a gel slab of 140 by 120 by 1.5 mm. The 30-ml gel mixture contained 7% acrylamide, 0.2% *N,N'* -methylenebisacrylamide, and 9.16 M urea in double-distilled deionized water. Ampholytes in the pH ranges of 5 to 7 (0.665 ml) and 3.5 to 10 (0.835 ml) were added to the mixture followed by 30 μl of freshly prepared 10% ammonium persulfate. The mixture was degassed under vacuum for 2 min, and then 20 μl of *N,N,N',N'*-tetramethylenediamine was added immediately before pouring. The upper buffer reservoir (cathode) contained 0.02 M NaOH, and the lower buffer reservoir contained 0.01 M H_3PO_4. The gel was prerun at 50 V for 15 min, 200 V for 15 min, and 300 V for 15 min, followed by 400 V for 30 min, all at room temperature (20°C). The samples were loaded into the preformed wells, and IEF was continued at 400 V (constant) for 18 to 24 h at 20°C.

The pH gradient in the gel was determined from macerated 5-mm cross sections of a lengthwise slice of the gel that were soaked for 24 h in 4 ml of double-distilled deionized water. The pH of the sections was determined with a Fisher model 230 pH meter. The data from 12 gels were pooled, and a best-fit line relating pH to distance was determined by linear regression analysis (level of confidence, >99%; correlation, 0.911). The gels were stained as described by Fairbanks et al. (1971) except that the Coomassie blue concentrations were decreased by 60%.

Chemicals

All chemicals were reagent grade, and most were obtained from Sigma Chemical Co. (St. Louis, Mo.). Acrylamide and *N,N'*-bisacrylamide were purchased from Eastman Kodak Co. (Rochester, N.Y.) and the ampholytes were from LKB (Fisher Scientific Co., Edmonton, Alberta). Other electrophoresis chemicals were obtained from Bio-Rad Laboratories (Mississauga, Ontario).

Results

Calcium Requirement for Competence

A. vinelandii strain UW1 grew well in competence medium (OFe+N) containing 0 to 20 mM $CaCl_2$. Over this range, the viable count per milliliter was not affected during the 20 h growth period. The cells grown without calcium, however, were not competent (Table 4). Competence increased with increased calcium content of the OFe+N medium until the usual Burk medium concentration of 0.5 mM was reached. Thereafter, an increased calcium concentration caused a decrease in competence.

When the calcium content of the cultures pregrown in less than 0.5 mM calcium was made to 0.5 mM, transformation competence increased dramatically (Table 4). When magnesium (0.1 to 10 mM) was added similarly to cells pregrown in calcium-limited competence medium (OFe+N OCa), the maximum transformation frequency obtained was 5.5×10^{-5}, an insignificant increase over the background frequency of 3.4×10^{-5}. Only strontium could be substituted for calcium, but recovery was only 50% of the 0.5 mM calcium value when 3 mM strontium (optimal) was used.

TABLE 4. The Effect of the Calcium Content of the Competence Induction Medium on Competence Development.

mM Ca^{++} in medium[a]	Nif Transformation Frequency	
	Control	+0.5 mM Ca^{++} (total)
0	0	2.4×10^{-4}
0.0005	1.8×10^{-6}	7.7×10^{-4}
0.005	1.3×10^{-5}	3.3×10^{-3}
0.05	3.3×10^{-5}	5.8×10^{-3}
0.5	4.2×10^{-3}	NA[b]
1.0	4.1×10^{-4}	NA
5.0	1.1×10^{-5}	NA
10.0	1.7×10^{-6}	NA
15.0	2.3×10^{-7}	NA
20.0	0	NA

[a] The cells were grown in OFe+N medium containing the mM $CaCl_2$ concentrations listed, for 20 h at 30°C.

[b] Not applicable.

Requirement for the Growth Supernatant for Recovery

When 0.5 mM $CaCl_2$ was added back to cells pregrown in OFe+N OCa medium, there was a rapid recovery of competence in the first 15 min followed by a continued but decelerating rate for at least 90 min (Fig. 1). When cells from the OFe+N OCa medium were washed twice with distilled water and then suspended in iron-limited Burk buffer containing 0.5 mM $CaCl_2$, they recovered competence at a slower rate and achieved only 3% of the activity obtained when the growth supernatant was present. These results suggested that de novo synthesis probably was not involved in the rapid recovery and that the culture fluid contained components that combined with calcium, allowing recovery, or that the washing procedure removed components from the cells, thereby preventing recovery, or both.

Cells pregrown in OFe+N OCa medium were washed successively with distilled water in an attempt to remove components required for competence. The washed cells were then allowed to recover in filter-sterilized OFe+N OCa growth supernatant or in iron-limited Burk buffer, both containing 0.5 mM $CaCl_2$. Washing the cells once had little effect on viability or competence recovery in either case (Table 5). Two or more washes decreased recovery significantly. Recovery was always greater in the growth supernatant than in the buffer, indicating that there were components in the growth supernatant that promoted recovery. Recovery in the growth supernatant, however, decreased with each successive wash, indicating that additional components required for competence also were being removed by washing.

Figure 1. Time required for competence recovery. Strain UW1 cells were pregrown in 0Fe+N 0Ca medium for 20 h at 30°C. The culture was assayed for competence at zero time, and then 0.5 mM $CaCl_2$ (final concentration) was added to 20 ml of the culture in a 50-ml flask (●). The flask was incubated at 30°C with shaking, and cells were removed at time intervals for transformation assays. The cells were pelleted from another 20 ml of the original culture and washed twice with distilled water. The washed cells were suspended in 20 ml of iron-limited Burk buffer containing 0.5 mM $CaCl_2$ (○), and recovery was similarly assayed.

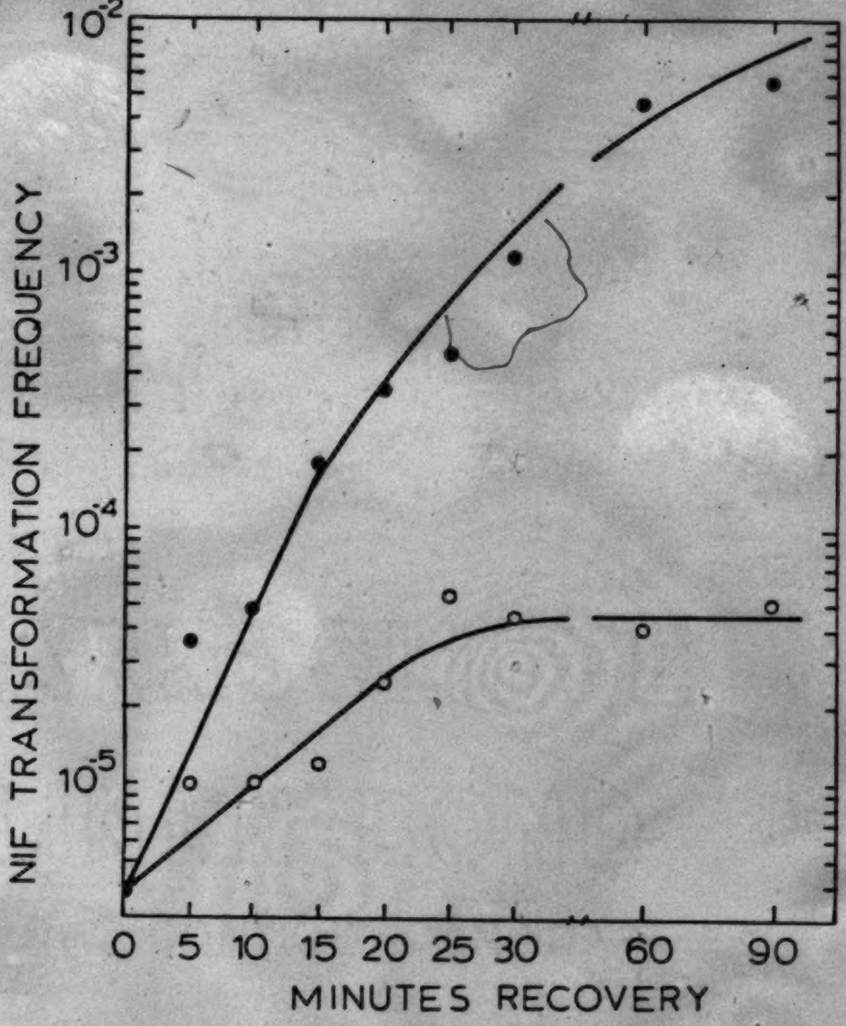

NIF TRANSFORMATION FREQUENCY
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
5
10
15
20
25
30
60
90
MINUTES RECOVERY

TABLE 5 . Effect of Washing on the Recovery of Competence.

Number of Distilled Water Washes	% of Original Viability[a]	Nif Transformation Frequency	
		Recovery in Supernatant[b]	Recovery in Buffer[c]
0	100	$5.5\text{x}10^{-3}$	$5.4\text{x}10^{-3}$
1	100	$3.7\text{x}10^{-3}$	$1.3\text{x}10^{-3}$
2	100	$1.1\text{x}10^{-3}$	$4.6\text{x}10^{-5}$
3	100	$1.0\text{x}10^{-4}$	$3.8\text{x}10^{-6}$
4	91	$1.4\text{x}10^{-5}$	$6.2\text{x}10^{-7}$
5	69	$4.2\text{x}10^{-6}$	ND[d]
6	60	ND	ND

[a] Viability was corrected for the loss of turbidity with each wash.

[b] Recovery was assayed as described in Figure 1, after resuspending the washed cell pellet in an original volume of filter sterilized OFe+N OCa growth supernatant and adding 0.5 mM $CaCl_2$, 30 min at 30°C.

[c] Recovery was assayed as described in Figure 1, after resuspending the washed cell pellet in an original volume of sterile iron-limited Burk buffer containing 0.5 mM $CaCl_2$, 30 min at 30°C.

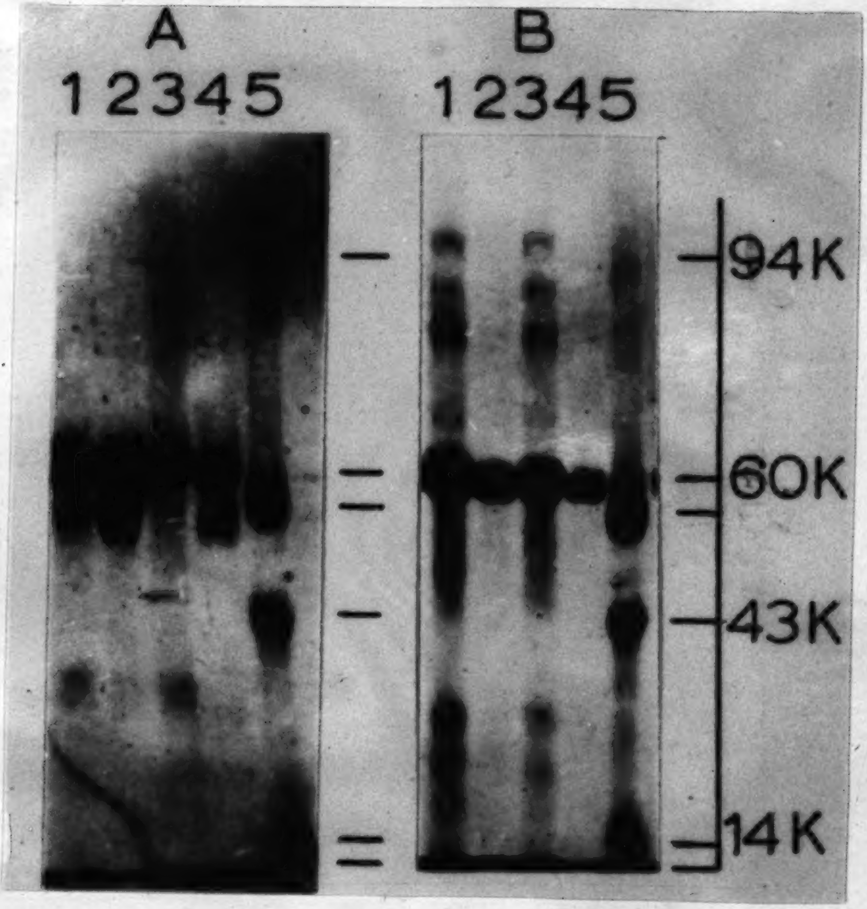
[d] None detected.

The recovery of cells pregrown in OFe+N OCa medium, washed twice with distilled water, also was promoted by noncompetent cell growth supernatants. Filter-sterilized BBGN and calcium-limited BBGN 20 h growth supernatants both allowed competence recovery when combined with the preceding cells and 0.5 mM $CaCl_2$ (transformation frequency, 2.0×10^{-3}). This was very similar to the levels of recovery obtained with sterile supernatants from OFe+N competence medium and OFe+N OCa medium (transformation frequencies of 2.8×10^{-3}, respectively). Therefore the growth supernatant aspect of recovery seemed to involve a component that was required for competence, but was not competence specific.

PAGE Analysis of Growth Supernatants and Distilled-Water Washes

Analysis of the four growth supernatants by SDS-PAGE revealed a major protein band with a molecular weight of 60,000 (Fig. 2A). This band also was stained by the periodic acid-Schiff reagent (data not shown), indicating that it was a glycoprotein, probably equivalent to that originally described by Schenk et al. (1977). The results clearly showed that relatively large amounts of glycoprotein were present in noncompetent cell supernatants. All of the supernatants also contained lesser amounts of a 47K band, and the calcium-limited supernatants contained a fainter-staining 36K band. When cells from the four growth conditions were washed six times with distilled water and the concentrated washings were similarly examined by SDS-PAGE, the 60K glycoprotein was the predominant band from competent OFe+N cells and noncompetent BBGN cells (Fig. 2B).

Figure 2. SDS-PAGE of culture supernatants and distilled water washes of cells. A 100-ml sample of each culture was treated as described in the text. (A) Concentrated culture supernatants (50 μl each) from: (1) BBGN OCa medium (63 μg of protein); (3) OFe+N OCa medium (45 μg of protein); (4) OFe+N medium (50 μg of protein); and (5) standards mixture. (B) Concentrated distilled-water washes of cells (25 μl each) from: (1) BBGN OCa medium (110 μg of protein); (2) BBGN medium (39 μg of protein); (3) OFe+N OCa medium (74 μg of protein); (4) OFe+N medium (39 μg of protein); and (5) standards mixture. The standards mixture contained 2.5 μg each of phosphorylase A (94,000), catalase (60,000), bovine gamma globulin (50,000), egg albumin (43,000), lysozyme (14,000), and RNase (13,700). The 60,000-dalton standard was absent from the gel A standards mixture, and the 94,000-dalton standard was absent from the gel B standards mixture.

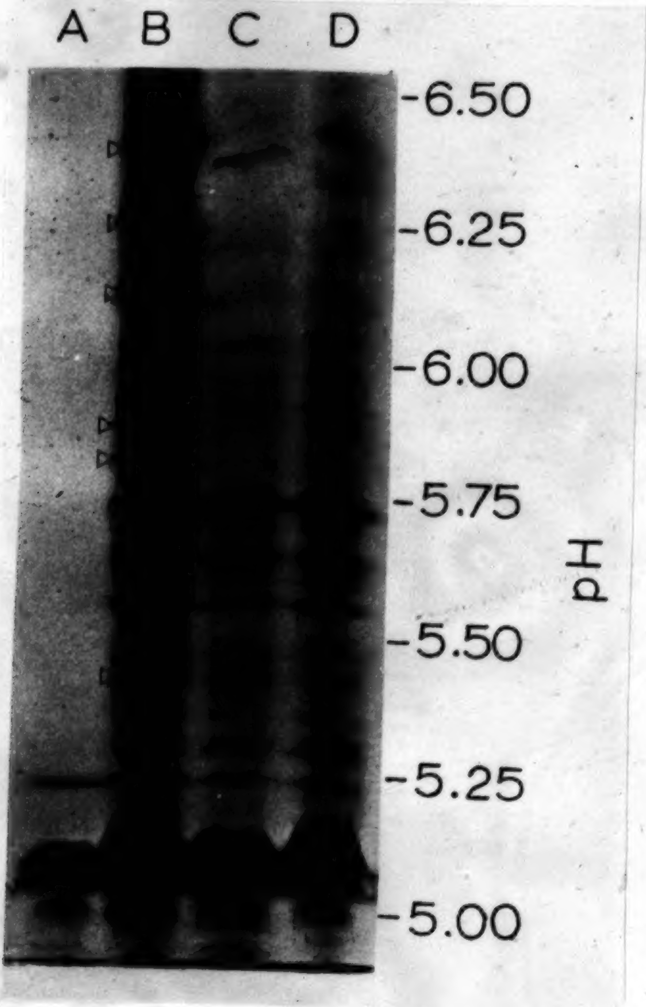

A
1 2 3 4 5
B
1 2 3 4 5
94K
60K
43K
14K

The growth supernatants from competent and noncompetent cells also were analyzed by IEF-PAGE to resolve competence-specific proteins that may have been released by calcium limitation. Very few proteins were released into the competent cell supernatant when calcium was present (Fig. 3A). All of these proteins were present in the other noncompetent cell supernatants (Fig. 3B-D). Calcium limitation allowed the release of a great variety of proteins, ranging from pI 4.9 to 6.5, whereas only the most acidic proteins were released by calcium-sufficient cells. Although no competence-specific proteins were observed, seven competence-dominant proteins appeared in the calcium- and iron-limited (potentially) competent cell supernatant (Fig. 3B).

Comparison of Glycoprotein and LPS in Promoting Competence Recovery

The pooled distilled water washings of 0Fe+N cells was used as a source of 60K glycoprotein. The glycoprotein was concentrated by precipitation with calcium as outlined in Experimental Procedures. Strontium could be substituted for calcium, but no precipitate formed with 20 to 25 mM magnesium. The concentrated glycoprotein gave a single band (60,000 molecular weight) by SDS-PAGE when 200 μg of protein was applied to the gel. Also present in the 0Fe+N supernatant was LPS as indicated by the thiobarbituric acid assay, so LPS also was concentrated as outlined in Materials and Methods. The purified glycoprotein promoted optimal recovery of competence when 24 μg of glycoprotein-protein was combined with 10^8 cells pregrown in 0Fe+N 0Ca medium and 0.5 mM $CaCl_2$ (transformation frequency, 7.4×10^{-4}; 25-fold greater than the background frequency). Purified LPS, however,

Figure 3. IEF-PAGE of proteins released into competent and noncompetent cell supernatants. Concentrated cell supernatants from OFe+N (A), OFe+N OCa (B), OFe+N (glutamate) OCa (C), and BBGN OCa (D) were loaded onto the gel such that the sample volume was standardized per 10^8 cells/ml in the original culture. Cells from the OFe+N medium were competent (transformation frequency, 1.6×10^{-2}), and cells from the OFe+N OCa medium recovered competence as shown in Fig. 1 (transformation frequency, 6.1×10^{-3}). Competence-dominant proteins (▷) are indicated in lane B.


A
B
C
D
-6.50
-6.25
-6.00
-5.75
-5.50
-5.25
-5.00
pH

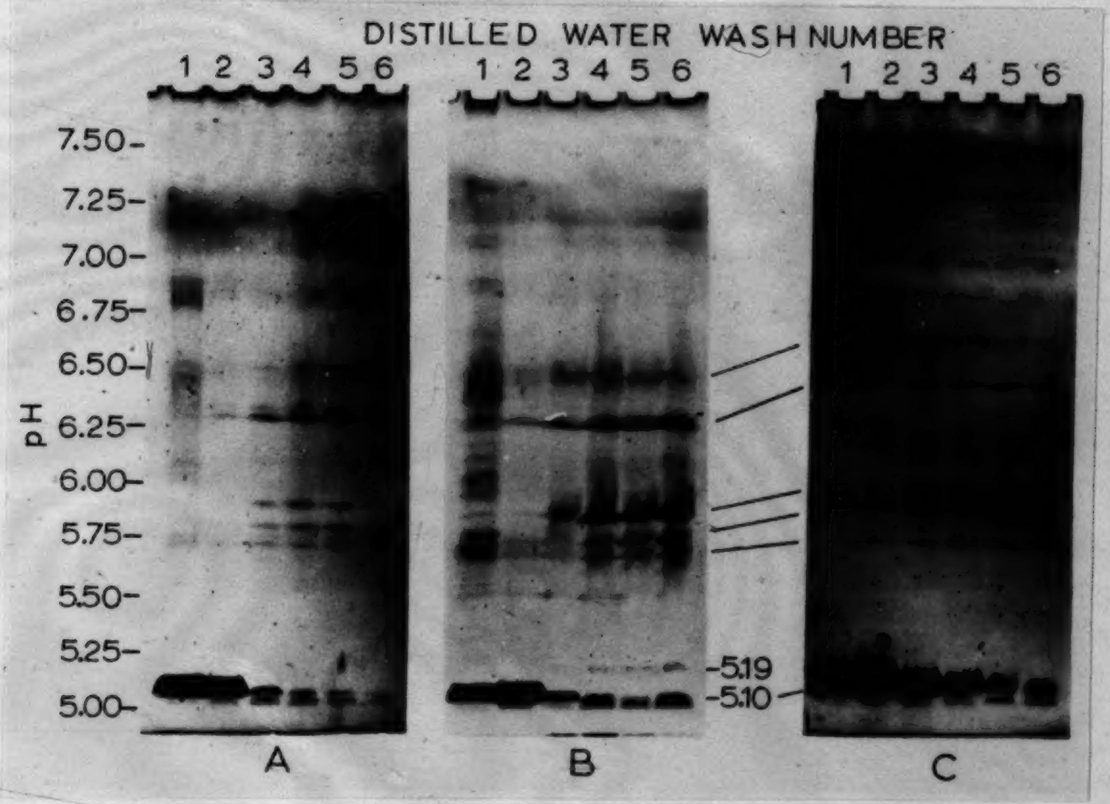
had little effect or inhibited recovery. When 0.6 µg of thiobarbituric acid-positive material was combined with 10^8 cells and 0.5 mM $CaCl_2$, the recovered transformation frequency was 2.2×10^{-5} (twofold greater than the background frequency). A 5-fold increase in LPS concentration resulted in a 10-fold decrease in transformation frequency. This was not due to a bacteriocidal effect of the preparation because the viable counts during the assay remained unchanged.

When competent strain UW1 cells were washed with distilled water, the transformation frequency decreased exponentially from 6.8×10^{-3} to 1.9×10^{-6} after six washes. The 60K glycoprotein was the major protein detected by SDS-PAGE (Fig. 2B). Washing competent cells six times with iron-limited Burk buffer neither decreased the competence of the cells nor released the 60K glycoprotein.

Removal of Competence-Specific Proteins by Washing

The data in Table 2 also suggested that components required for competence were removed from the OFe+N OCa-pregrown cells by washing. The six distilled water washes were each analyzed by IEF-PAGE to determine if unique components were removed with successive washes. To determine which of these components were competence specific, the IEF-PAGE patterns were compared with those of six consecutive washes of noncompetent cells grown in OFe+N OCa containing glutamate rather than ammonia as a nitrogen source. Equal volumes of each wash were loaded onto the gels in Fig. 4A and B to allow a visual comparison of the relative amount and variety of proteins removed. The results indicated that the major protein removed by the first three washes in both cases

Figure 4. IEF-PAGE comparison of distilled-water washes from competent and noncompetent cells. Approximately 3.0 x 10^{11} cells were treated for gels A and B; 4.6 x 10^{11} cells were treated for gel C. Protein values are given in the brackets. (A) Noncompetent cells grown in OFe+N (glutamate) OCa; washes 1 (98 μg), 2 (68 μg), 3 (33 μg), 4 (25 μg), 5 (13 μg), and 6 (13 μg). (B) Competent cells grown in OFe+N OCa; washes 1 (125 μg), 2 (25 μg), 3 (38 μg), 4 (50 μg), 5 (38 μg), and 6 (50 μg). (C) Noncompetent cells grown in OFe+N OCa; washes 1 to 6 contained 200 μg of protein.


DISTILLED WATER WASH NUMBER
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
pH
7.50
7.25
7.00
6.75
6.50
6.25
6.00
5.75
5.50
5.25
5.00
5.19
5.10
A
B
C

was the glycoprotein (pI 5.10). The position of the glycoprotein in these gels was confirmed by using the concentrated glycoprotein preparation (Appendix 1). After four to six washes, the OFe+N OCa cells released a protein (pI 5.19) not observed in the noncompetent cell washes. Although this was the only competence-specific protein observed in these gels, it does not exclude (some of) the other proteins from competence involvement. Because different amounts of protein were released by each wash and a different nitrogen source was used to prevent competence development, it was possible that the pI 5.19 band was not competence specific. Therefore, another culture of OFe+N OCa cells whose recovery of competence was very low (transformation frequency, 4.0 x 10^{-6}) compared with that of the previous culture (transformation frequency, 1.6 x 10^{-4}) was similarly washed and analyzed. The pI 5.19 band was not evident in these cells, even though more than five times the amount of protein was analyzed in washes 4, 5, and 6 (Fig. 4C). Thus, the pI 5.19 band appeared to be competence specific.

Discussion

Although *A. vinelandii* cells grew well over a 24-h period in calcium-limited competence medium, they did not become highly competent. Optimal competence required the presence of 0.5 mM calcium. The calcium requirement for competence was therefore 10-fold greater than the 50 μM level required for optimal growth (Barnes et al., 1978). This observation coupled with the known magnesium requirement for transformation (Page and von Tigerstrom, 1979) confirms that both of these cations are required in the *Azotobacter* transformation system.

The calcium requirement for competence development is unlikely to serve the same purpose as the 100 or 50 mM calcium treatments used to render *Staphylococcus aureus* or *Escherichia coli* K-12 and *Pseudomonas aeruginosa*, respectively, competent (Cosloy and Oishi, 1973; Mercer and Loutit, 1979; Sjostrom et al., 1972). Unlike these other systems, magnesium would not substitute in the calcium requirement and 20 mM calcium effectively prevented competence development in *Azotobacter*. Although both calcium and magnesium are involved in plasma membrane (Fill and Branton, 1969) and outer envelope stability (Stan-Lotter et al., 1979; Yem and Wu, 1978), calcium may form specific protein and LPS complexes which affect envelope permeability (van Alphen et al., 1978). Similarly, calcium or strontium, but not magnesium, is required for the integrity and attachment of superficial cell wall layers (Beveridge and Murray, 1976; Buckmire and Murray, 1976; Glauert and Thornley, 1973). Further evidence of the functional partitioning of calcium is seen in its requirement for the epimerization of capsular polyuronic acids and cyst coat maturation in *Azotobacter* (Haug and Larsen, 1971; Page and Sadoff, 1975).

Although the exact location of the 60K glycoprotein remains to be shown, these present data and those of Schenk et al. (1977) indicate that it is probably attached to the cell surface. It is easily removed by washing cells in distilled water and is the only component removed from cells with intact envelopes (BBGN or OFe+N medium). Washing these cells with buffer, however, does not release the glycoprotein, suggesting that the ionic strength of the wash is critical. These results parallel those of Buckmire and Murray (1976), who investigated the removal of the superficial layers of the *Spirillum serpens* envelope. Addition of calcium to calcium-limited cells in solutions containing glycoprotein resulted in a rapid recovery of competence. Strontium, but not magnesium, substituted for calcium in this activity. This rapid recovery could have been the result of the reassembly of a surface component. Preliminary results show that the cell surface binding of ruthenium red is increased when glycoprotein is removed and no enzymatic activity has been detected for the glycoprotein (W.J. Page, unpublished data). The strongly acidic pI of this component coupled with its affinity for calcium (exploited in its concentration from culture fluids) may allow the glycoprotein to mask negative charges on the cell surface through salt-bridge association. The neutralization of some surface charges may be a prerequisite for DNA association with the cell surface and its attaining a DNase-insensitive state. These possibilities await experimental confirmation.

The overall effect of calcium in recovery of competence, however, is probably more pleiotropic than simple reassembly by glycoprotein onto the cell surface. For example, there appears to be a specific calcium requirement for the transport of DNA across the competent pneumococcal plasma membrane (Seto and Tomasz, 1976). Similarly, this may be a necessary, but not sufficient, condition for *Azotobacter* transformation because calcium alone increased but did not restore competence completely. The outer envelope integrity also seemed to be weakened by calcium limitation. These cells released more proteins into their growth supernatant and into their distilled water washes than calcium-sufficient cells. The apparent increased permeability of the calcium-limited cell envelope, however, was exploited to expose competence-dominant proteins and a competence-specific protein (pI 5.19).

Acknowledgments

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Note Added in Proof

The apparently competence-specific protein (pI 5.19) was also absent from distilled water washes of noncompetent cells prepared by growth in Burk medium lacking calcium (Appendix 2).

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IV. Heat-Sensitivity of Genetic Transformation of *Azotobacter vinelandii*

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Summary

Heating competent *Azotobacter vinelandii* at 37°C or 42°C resulted in a total loss of competence with no loss of viability. The transformation process was relatively insensitive to heating at either temperature once DNase-resistant DNA binding was nearly complete. Although competent and 42°C-treated cells bound equivalent amounts of [^{32}P]DNA in a DNase-resistant state, no donor DNA marker (*nif*) or radioactivity were detected in the envelope-free cell lysate of heated cells, suggesting that DNA transport across the cell envelope was a heat-sensitive event. Competence was reacquired in a 42°C-treated culture after 2 h of incubation at 30°C by a process which required RNA and protein syntheses. The release of a surface glycoprotein, required for competence, from cells treated at 42°C occurred in an insufficient amount to account for the total loss of competence. Recovery of competence in 42°C-treated cells and further transformation of competent cells were prevented by the exposure of cells to saturating amounts of

transforming DNA. However, further DNase-resistant DNA binding, still occurred suggesting that there were two types of receptors for DNase-resistant DNA binding in competent *A. vinelandii*. DNase-resistant DNA binding was dependent on magnesium ion and at least one receptor type did not discriminate against heterologous DNA.

Introduction

Competence for bacterial transformation designates a physiological state in which cells are able to bind exogenous DNA and transport it across the cell envelope. Competence defined as the ability of cells to bind DNA in a DNase-resistant state (Lerman and Tolmach, 1957) is not applicable to *Azotobacter vinelandii* as both transformable and nontransformable cells share equally in this ability (this study). One approach to the study of physiological aspects of *Azotobacter* competence is to determine the basis for treatments which are detrimental to competence (Page and Doran, 1981). It has been shown that heating competent *A. vinelandii* at 37°C or 42°C reduces competence (Page and von Tigerstrom, 1979). An envelope glycoprotein of apparent-molecular-weight 60,000 (60K), which is required for competence (Page and Doran, 1981), is removed from the cells by distilled water washing at 38°C (Schenk and Earhart, 1981). We report here that 60K glycoprotein removal is primarily effected by washing cells with distilled water and that the amount released upon heating cells in buffer is insufficient to account for competence loss at 37 to 42°C.

The treatment of competent *Haemophilus influenzae* at 42°C results in a reduction in the ability of cells to bind DNA in a DNase-resistant state (Barnhart and Herriott, 1963). Heating competent *Streptococcus pneumoniae* at 35 to 40°C has the same effect and this ability is regenerated at 30°C by a process requiring protein synthesis (Lacks and Greenberg, 1973). Similarly, heating competent *S. sanguis* (Challis) at 48°C for 20 min decreases the ability of cells to irreversibly bind DNA (Ravin and Ma, 1972) and also reduces the ability of cells to discriminate against heterospecific DNA and low-efficiency homospecific markers (Deddish and Ravin, 1974). Competent *Bacillus subtilis* is unable to bind DNA in a DNase-resistant state following brief heating at 50°C (McCarthy and Nester, 1969) and donor marker survival is also sensitive to heating (McCarthy and Nester, 1969; McDonald, 1971). The results of this investigation indicate that competent *A. vinelandii* heated at 42°C for 20 min is unable to transport donor DNA across the cell envelope but are fully capable of binding DNA in a DNase-resistant state. The survival and expression of newly transported donor DNA marker are insensitive to heating at 42°C.

(Preliminary results of this study were presented at the 81st Annual Meeting of the American Society for Microbiology, 1 to 6 March 1981, Dallas, TX.)

Experimental procedures

Bacterial strains and growth conditions

The transformation recipient *Azotobacter vinelandii* OP strain UW1 (Nif^- $capsule^-$) and the parental strain UW (Nif^+ $capsule^-$) were provided by W. Brill (University of Wisconsin, Madison). *A. vinelandii* ATCC 12837 strains 113 (Nif^+ rifampicin-resistant) and 114 (Nif^+

streptomycin-resistant) and the diazotrophs *Azotobacter chroococcum* ATCC 7486, *Azotobacter paspali* ATCC 23368, *Azotobacter beijerinckii* ATCC 19360, *Azomonas macrocytogenes* ATCC 12335, *Azomonas insignis*, *Beijerinckii indica* ATCC 12523, *Rhizobium meliloti* 17A, *R. trifolii* CC10, *Klebsiella pneumoniae* ATCC 13883 and *Clostridium pasteurianum* NCIB 9486 were used as sources of donor *nif* DNA. *C. pasteurianum* was grown under anaerobic conditions at 37°C on modified Burk medium containing 2% sucrose and 2 μg biotin/liter. *K. pneumoniae* was grown at 37°C on nutrient agar (Difco) containing 1% glucose. *Rhizobium* species were grown in yeast extract agar containing 1% glucose at 30°C. *A. paspali* and *A. beijerinckii* were grown at 30°C on N-free Burk medium containing 1% sucrose as the C source. Other Nif$^+$ strains were grown at 30°C on N-free Burk medium (Page and Sadoff, 1976). Strain UW1 was grown on Burk medium containing 1.1 g of ammonium acetate/liter. Liquid cultures were shaken at 170 rpm in a water bath gyrotory shaker (model G-76; New Brunswick Scientific Co., New Brunswick, N.J.).

Crude lysate DNA preparation

Crude lysate DNA was prepared by suspending cells from slant cultures in 15 mM saline-15 mM sodium citrate buffer, pH 7.0 (SSC), containing 0.05% sodium dodecyl sulfate (SDS) and heating at 60°C for 60 min (Page and Sadoff, 1976). Heating was extended for up to 6 h for organisms other than *A. vinelandii*. In some cases DNA was further purified by phenol extraction, RNase treatment and removal of oligoribonucleotides and capsular material as described by Marmur (1961). DNA concentration was estimated using a modified (Giles and Meyers, 1965) Burton (1956) assay.

Transformation assay

Competence for genetic transformation was induced in strain UW1 by 18 to 22 h of growth in Fe-limited Burk medium as described previously (Page and von Tigerstrom, 1978). Except where noted, competent cells were transformed in Burk buffer, pH 7.2, containing 8 mM $MgSO_4$ (transformation assay buffer [Page and von Tigerstrom, 1979]) using 10 μg of crude lysate DNA per 10^8 cells. This amount of DNA was approximately 10 times that required to saturate the cells. Similarly, [^{32}P]DNA was used at 2 to 3 times the saturating concentration. The reaction was stopped by the addition of DNase 1 (final concentration, 4 μg/ml) and incubation at 30°C for 5 min.

Cells to be transformed a second time with additional DNA were centrifuged and resuspended in one volume of transformation assay buffer and then treated with trypsin to degrade residual DNase followed by trypsin inhibitor (final concentrations, 4 μg/ml), each for 10 min at 30°C. The cells were collected again, resuspended in transformation assay buffer and exposed to the second DNA species. Competence and competence recovery in 42°C-treated cells were not sensitive to trypsin at this concentration.

Nif$^+$ transformants were detected by plating cells directly on N-free Burk medium. Rifampicin-resistant and streptomycin-resistant transformants required 20 h of incubation in Burk medium for phenotypic frequency stabilization (Page and von Tigerstrom, 1979) before plating on selective Burk medium containing 20 μg/ml rifampicin or streptomycin. Transformation frequency was calculated as the number of transformants detected per total number of viable cells plated on selective medium. All determinations were made at least twice.

Preparation of sterile competent-culture supernatant

It was often necessary to remove competent cells from their original growth medium after a specific treatment and resuspend the cells for subsequent incubation. Resuspending competent cells in fresh Fe-limited Burk medium resulted in lower levels of competence and poorer competence recovery than resuspending cells in filter-sterilized, original growth supernatant (Appendix 3). In all instances, either competent culture supernatant or 42°C-treated competent culture supernatant served equally well as a resuspension medium.

Concentration of culture supernatants and distilled water washes of competent cells

Culture supernatant fluids were concentrated for the analysis of glycoprotein released by competent cells, and Ca-limited competent cells, as described previously (Page and Doran, 1981). Distilled water washes of competent cells performed at either 30°C or 42°C also were concentrated as previously described (Page and Doran, 1981).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) under the conditions described previously (Page and Doran, 1981). Proteins were stained as described by Fairbanks et al. (1971) using Coomassie blue R250 in isopropanol and acetic acid.

Time of expression of nitrogenase activity in newly transformed cells

Cells from a competent culture of strain UW1 were washed twice by centrifugation using 0.5 volumes of Fe-limited N-free Burk medium to remove excess ammonia. The cells were resuspended to the original volume in filter-sterilized culture supernatant obtained from strain UW grown 20 h in Fe-limited N-free Burk medium. A 5-ml amount of this cell suspension was dispensed into each of twelve 10-ml Erlenmeyer flasks and the cultures were incubated at 30°C for 4 h to starve the competent cells for nitrogen (Appendix 4). The cultures were then transferred to centrifuge tubes containing 1.5 ml of strain 113 crude lysate DNA (60 μg DNA/ml) and 18 ml of transformation assay buffer. After 20 min of incubation at 30°C the reaction was stopped with 1.0 ml of 100 μg/ml DNase 1. The cells were harvested by centrifugation, resuspended in 5 ml of Fe-limited N-free Burk medium and incubated in 10-ml Erlenmeyer flasks at 30°C for expression of nitrogenase activity. At 20 min intervals, beginning immediately, flasks were sealed with serum stoppers and nitrogenase activity was measured by the acetylene-ethylene reduction assay (Hardy et al., 1968) as previously described (Page, 1982). Aliquots of each culture were plated to determine the viable number and frequency of Nif^+ transformants. Nitrogenase specific activity was calculated as nmole ethylene formed/h/10^8 Nif^+ transformants.

Recovery of donor DNA from transformed cells

Transformation assays were set up in five pairs of 15 ml conical centrifuge tubes, each containing 3.0 ml of transformation assay buffer and 50 μg of strain 113 crude lysate DNA. One tube of each pair received 0.5 ml of competent strain UW1 culture and the other received 0.5 ml of the same culture previously treated at 42°C. After 1, 10, and 30 min incubation at 30°C, DNA binding was stopped by the addition of 1 ml of 20 μg/ml DNase 1 and each pair of tubes was placed on ice. DNase was digested with trypsin as described above but incubations were for 2 min at 22°C, a temperature at which transformation occurs very poorly (Page and von Tigerstrom, 1979). The cells were collected by centrifugation at 4°C and crude lysates were prepared. Cell envelopes were removed by centrifugation at 20,000 rpm for 60 min and the supernatant was decanted and stored at 4°C. DNA binding in the remaining two pairs of tubes was stopped after 30 min. DNase was inactivated as described above using 5-min incubation periods at 30°C. The cells were collected by centrifugation, resuspended in 0.5 ml of sterile culture supernatant and incubated with shaking at 30°C in 10-ml Erlenmeyer flasks. Envelope-free cell lysates were prepared from these samples 60 and 90 min after the initiation of transformation.

The envelope-free cell lysates (0.5 ml) were assayed for transforming activity by mixing each with 1 ml of freshly prepared strain UW1 competent cell suspension in 6 ml of transformation assay buffer. The reaction was stopped after 20 min incubation at 30°C by the addition of 1.0 ml of 20 μg/ml DNase 1. As the number of Nif$^+$ transformants was expected to be small the recipient cells were concentrated 7.5 fold prior to plating on selective medium.

Preparation of purified [^{32}P]DNA

Crude lysate DNA was prepared from strain UW1 grown for 2 days in modified Burk medium containing 1 mM phosphate buffer, pH 7.2, and 2.5 mCi of [^{32}P]phosphate (55% of which became incorporated). The crude lysate was treated with proteinase K and further purified as described (Marmur, 1961). [^{32}P]DNA was purified free of residual contaminating [^{32}P]oligoribonucleotides by 4.5 h electrophoresis at 50 V in 0.5% agarose - 20 mM Tris (hydroxy-methyl) aminomethane (Tris)-2 mM EDTA-50 mM sodium acetate, pH 7.8, (TEA) gels. [^{32}P]DNA detected by autoradiography using Kodak X-OMAT X-ray film was extracted by gel dissolution in KI and DNA binding to hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) (Smith, 1980). The HAP-[^{32}P]DNA was washed free of agarose and KI as described (Smith, 1980) except that HAP-[^{32}P]DNA was not formed into a column as better recoveries of [^{32}P]DNA were obtained by batch treatment. Treatment with 1 M sodium phosphate - 1 mM EDTA released 20 to 30% of [^{32}P]DNA which was dialysed twice in 48 h against 4 L of SSC at 4°C. Samples concentrated by dialysis against polyethylene glycol (PEG 20,000) were analyzed by agarose gel electrophoresis and all radioactivity detected by autoradiography was present in high molecular weight DNA. Treatment with DNase 1 rendered 98% of the radioactivity soluble in cold 10% trichloroacetic acid. The specific activity of the [^{32}P]DNA preparation varied with different batches and the age of the preparation. Nonradioactive purified DNA also was prepared by this method.

[^{32}P]DNA binding and uptake

Cells from a competent strain UW1 culture and from a 42°C-treated formerly-competent culture were concentrated 10 fold into 0.5 ml of sterile competent culture supernatant and mixed with 3 ml of transformation assay buffer containing 0.5 ml of [^{32}P]DNA. After 10 min of incubation the cells were pelleted and washed once by centrifugation with 4 ml of transformation assay buffer, resuspended in the same, and treated with DNase 1 for 5 min. The amount of unabsorbed [^{32}P]DNA and the amount of radioactivity released by DNase treatment of cells were determined by adding 4 ml of aqueous sample to 6 ml of Monophase 40 liquid scintillation fluor (Packard Instrument Co. Inc., Downers Grove, Illinois) and measuring radioactivity with a Searle model 6880 liquid scintillation counter. Residual DNase in the transformed cell suspension was digested with trypsin which in turn was inactivated with trypsin inhibitor as described above. The cell pellet was resuspended in 4 ml of sterile culture supernatant. A crude lysate (total volume 4 ml) was prepared from 0.5 ml of this suspension and was used to determine the amount of [^{32}P]DNA bound to cells in DNase-resistant state. The remaining 3.5 ml of cell suspension was shaken for 2 h in a 10-ml Erlenmeyer flask to allow completion of the transformation process. ^{32}P released into the culture supernatant was measured. The location of bound [^{32}P]DNA was analysed further after spheroplasts were formed by resuspending cells in 3.5 ml of 50 mM Tris-50 mM EDTA-10% (w/v) sucrose, pH 8.0, and incubating for 60 min at 30°C with 100 μg/ml lysozyme. Pelleted spheroplasts were lysed by twice resuspending them in 5 ml of distilled water. The lysates were pooled and centrifuged at 5000 rpm, 10 min, to collect unbroken cells which, typically, were absent. The lysate was then centrifuged at 20,000 rpm for 70 min to obtain the spheroplast membranes and the radioactivity in this pellet and in the supernatant was measured.

The spheroplast lysate was analysed to determine whether all radioactivity was present as high molecular weight DNA. DNA and RNA precipitated by adding 10 ml of lysate to 30 ml of 95% ethanol and placing at -20°C for 24 h were collected by centrifugation at 4,000 rpm for 60 min and the amount of soluble ^{32}P remaining in the supernatant was measured. The ethanol-precipitable material was subjected to electrophoresis on a 0.4% agarose TEA gel for 10.5 h at 20 V. Lambda cI857 DNA was used as a molecular weight indicator. The gel was stained with ethidium bromide and DNA and RNA bands were detected by fluorescence (Sharp et al., 1973). [^{32}P]-labeled material was detected by autoradiography for 9 days at 70°C as described earlier but using Dupont Cronex Lightning-Plus intensifying screens.

Chemicals

All fine chemicals were reagent grade and most were obtained from Fisher Scientific Co. (Edmonton, Alberta) except enzymes and trypsin inhibitor which were from Sigma Co. (St. Louis, MO.). Agarose was supplied by Mandel Scientific Co. (Calgary, Alberta). Carrier free [^{32}P] phosphoric acid in aqueous solution, was supplied by Amersham (Arlington Hts, Il). Acrylamide and N,N'-methylene bisacrylamide were from Eastman Kodak Co. (Rochester, NY). All other electrophoresis chemicals were obtained from BioRad Laboratories (Mississauga, Ontario).

Results

Loss of competence at elevated temperature and competence recovery

Holding a competent culture of *A. vinelandii* at 37°C or 42°C resulted in an exponential loss of competence (Fig. 5). Although competence loss at 37°C occurred at a slower rate and after a lag of 20 min, both treatments resulted in a complete phenotypic loss of competence. The treatment of cells at either temperature had no effect on viability and holding competent cells at 30°C had no effect on competence.

Transformable cells were regenerated in a 42°C-treated culture by incubation at 30°C (Fig. 6). The maximum level of competence attained was not always as high as that prior to heating. This probably reflected the normal decay of competence which occurred after an extended period of incubation in Fe-limited medium (Page and von Tigerstrom, 1978). There was no change in the number of viable cells during the period of competence recovery. The recovery of competence appeared to require RNA and protein syntheses because recovery was delayed when cells were treated with rifampicin or chloramphenicol (Fig. 6). Rifampicin and chloramphenicol at these concentrations had no effect on the maintenance of competence in an unheated culture.

It has been reported that a 60,000 molecular weight (60K) envelope glycoprotein required for competence (Page and Doran, 1981) was completely removed from *A. vinelandii* by washing with distilled water at 38°C but not at 33°C (Schenk and Earhart, 1981). It was possible, therefore, that synthesis of 60K glycoprotein was the step necessary for competence recovery in 42°C-treated cells. Although the culture

Figure 5. Loss of competence at elevated temperature. Competent strain UW1 culture was held at 30°C (△), 37°C (□) or 42°C (○). At time intervals aliquots of the cells were removed, equilibrated to 30°C and assayed for transformation frequency (closed symbols) and viable number (open symbols).

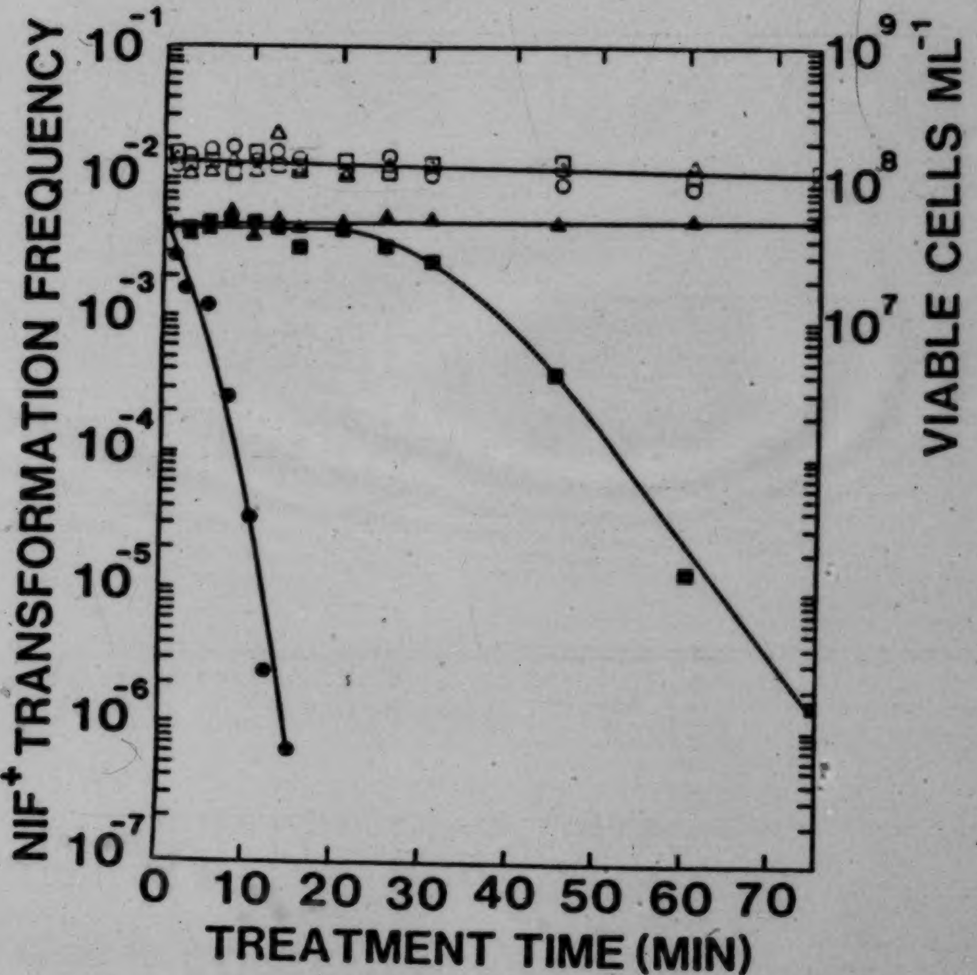
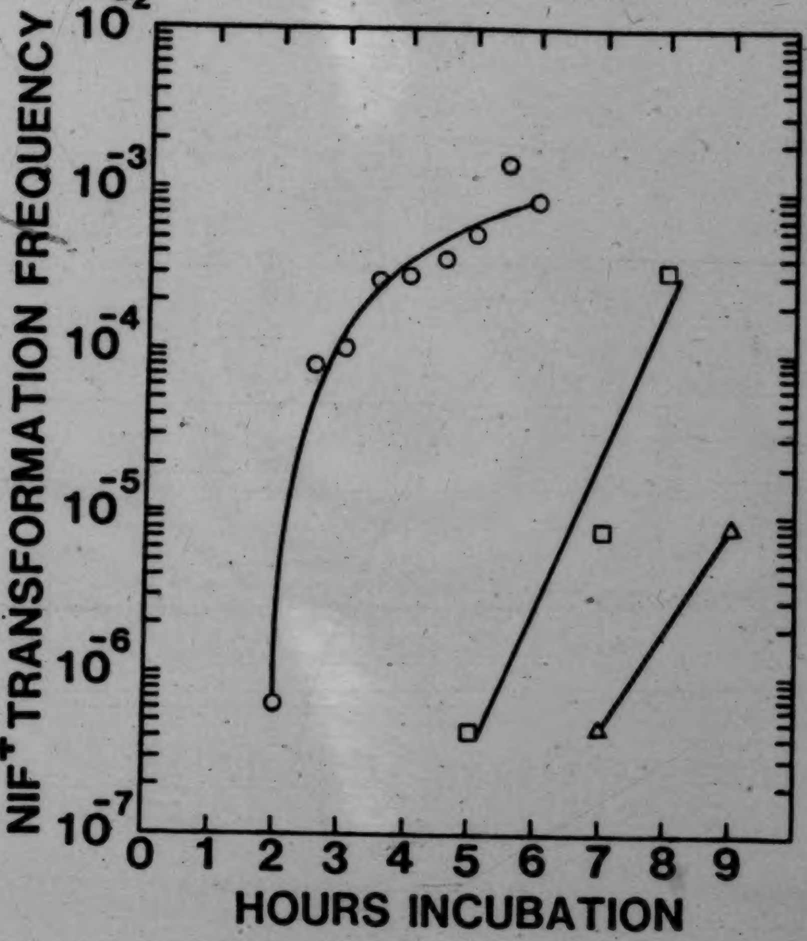

NIF$^+$ TRANSFORMATION FREQUENCY
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
0
10
20
30
40
50
60
70
TREATMENT TIME (MIN)
10^9
10^8
10^7
VIABLE CELLS ML^{-1}

Figure 6. Recovery of competence in 42°C-treated cultures. A culture of competent strain UW1 (Nif transformation frequency 1.7 x 10^{-3}) was held at 42°C for 25 min to eliminate competence. Aliquots of the 42°C-treated culture were incubated with chloramphenicol (25 μg/ml, final concentration,□), rifampicin (1 μg/ml, final concentration,△), or no further additions (○). After 30 min incubation at 30°C the cells were harvested by centrifugation and were resuspended in the original volume of original competent culture supernatant. Incubation was continued and at time intervals samples were removed for transformation assay.


NIF⁺ TRANSFORMATION FREQUENCY
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
0
1
2
3
4
5
6
7
8
9
HOURS INCUBATION

supernatant of 42°C-treated competent culture contained somewhat more 60K glycoprotein than competent culture supernatant it was much less than was removed by washing competent cells with distilled water at 30°C (Fig. 7). Distilled water washing of cells resulted in a five-fold reduction of competence (transformation frequency 2.4 x 10^{-4}) whereas 42°C-treated cells were noncompetent. Cells pregrown in Ca-limited competence medium developed a very low level of competence (transformation frequency 1.8 x 10^{-6}) and released a similar large amount of glycoprotein into the culture supernatant (Fig. 7). Although 60K glycoprotein loss has been correlated with competence loss (Page and Doran, 1981), clearly, glycoprotein release from 42°C-treated competent cells was insufficient to account for the complete loss of competence observed. These data also indicated that the release of glycoprotein from cells was mediated primarily by washing with distilled water and not by treatment at 42°C.

Effect of elevated temperature on transformed cells

Competent cells exposed to a 10-fold excess of donor DNA became nearly saturated with DNA bound in a DNase-resistant form after 5 to 10 min (Fig. 8). DNase-resistant DNA binding was prevented by DNase added 1 sec but not 2 sec after DNA addition. Shifting cells to 37°C during this period of rapid DNase-resistant DNA binding had little or no effect on the transformation frequency whereas heating at 37°C after this time produced a small but consistently observed enhancement. Shifting the transformation assay to 42°C after DNase-resistant DNA binding produced only a small but consistent decrease in transformation frequency.

Figure 7. SDS-PAGE of culture supernatants and 30°C distilled water washes of competent cells. All samples were concentrated to a standard volume per 10^8 cells/ml in the original culture and 50 μl of each was added to the gel. Lanes 1, 3 and 4 contained concentrated culture supernatants from strain UW1 grown in (1) Ca- and Fe-limited Burk medium, (3) Fe-limited Burk medium, and (4) pregrown in Fe-limited Burk medium and treated at 42°C for 25 min. Lane 2 contained concentrated 30°C distilled water washes of competent cells. The molecular weight standards, phosphorylase A (94,000), bovine gamma glubulin (50,000) and ovalbumin (43,000), were used to determine the molecular weights shown.

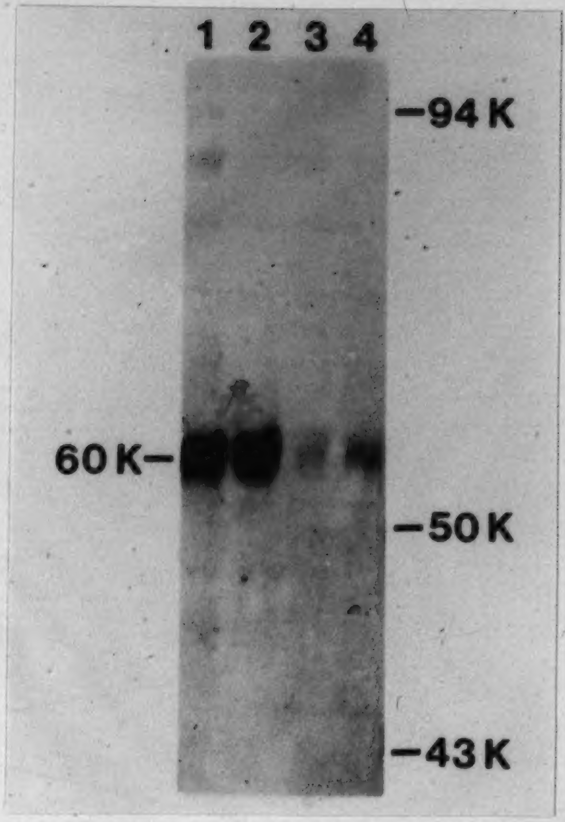
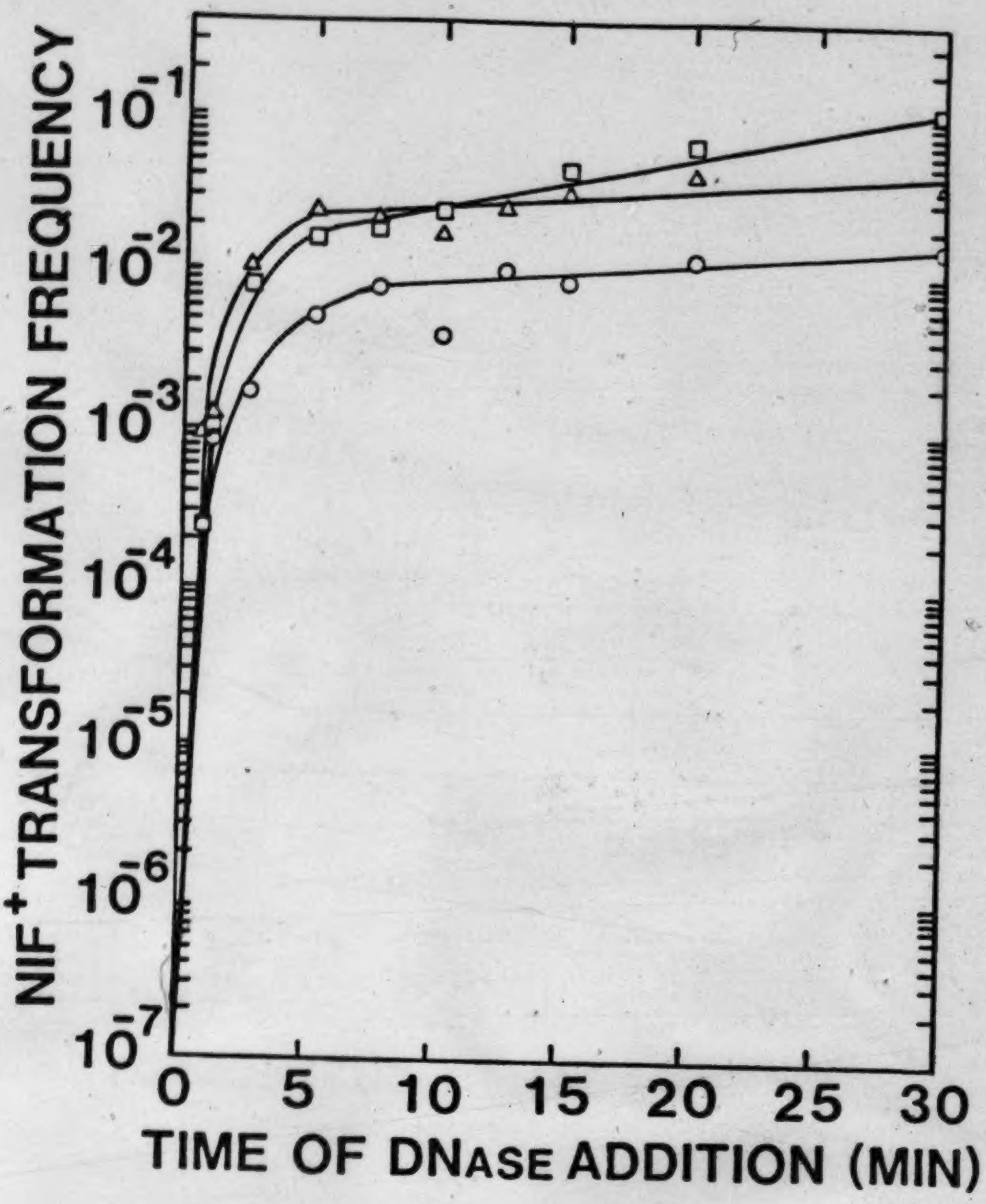

1
2
3
4
94 K
60 K
50 K
43 K

Figure 8. Treatment of competent cells at 30°C, 37°C or 42°C following DNase-resistant DNA binding. At zero time crude lysate DNA was mixed with strain UW1 to initiate transformation at 30°C. At time intervals thereafter DNase 1 was added and the assay mixtures were immediately transferred to preheated tubes at 30°C (△), 37°C (□) or 42°C (○) and held at this temperature for 30 min prior to plating on selective medium.


NIF⁺ TRANSFORMATION FREQUENCY
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
0
5
10
15
20
25
30
TIME OF DNASE ADDITION (MIN)

The failure to detect transformants in a 42°C-treated formerly competent culture could not be attributed to a temperature-sensitive event in the subsequent steps of the transformation process. Newly acquired nitrogenase genes in transformed, nitrogen-starved UW1 were expressed 60 to 80 min after the addition of transforming DNA (Fig. 9A). Neither the survival of the donor DNA *nif* marker in the recipient cell nor expression of the *nif* genes (Fig. 9B) was particularly sensitive to heating at 42°C.

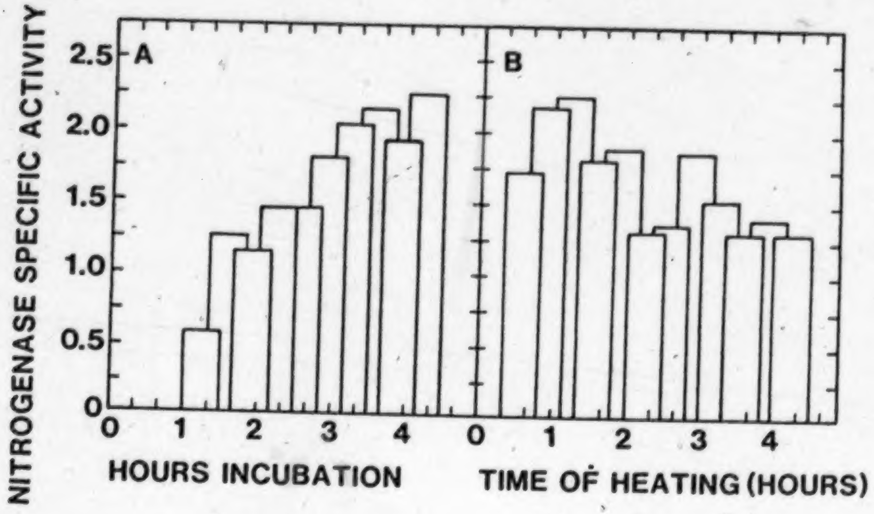
Recovery of *nif* marker from recipient cells

Donor (*nif*) activity was recovered from transformed cell envelope-free lysates 1 min after mixing donor DNA with the strain UW1 recipient (transformation frequency 5.8×10^{-6}). The Nif$^+$ transforming activity of the competent cell lysates increased with time during the 30 min exposure of cells to donor DNA and during the 60 min further incubation after the addition of DNase (maximum frequency 2.4×10^{-4}). The second recipient was transformed at a frequency of 3.5×10^{-2} when exposed to a similar concentration of original donor DNA. No Nif$^+$ donor activity was recovered from similarly prepared 42°C-treated cell lysates. These results suggested that DNA uptake was the heat-sensitive event.

Binding and uptake of [^{32}P]DNA

Competent cells, pretreated at 42°C, bound equivalent amounts of [^{32}P]DNA as unheated competent cells ($P = 0.05$). Two-thirds of the [^{32}P]DNA bound to either cell type was insensitive to DNase. The

Figure 9. Effect of 42°C heating on *nif* marker survival and *nif* expression in transformed cells. Strain UW1 was assayed for nitrogenase activity at 20 min intervals after the addition of Nif$^+$ DNA to a competent (Nif$^+$ transformation frequency, 1.6 x 10^{-2}), N-starved culture (A). Similar N-starved cells were incubated for 20 min with donor DNA then shifted to 42°C for 30 min at 20 min intervals. After treatment at 42°C the cells were transferred to 30°C to give a total incubation of 5 h before nitrogenase activity was measured (B).


NITROGENASE SPECIFIC ACTIVITY
A
B
2.5
2.0
1.5
1.0
0.5
0
0
1
2
3
4
0
1
2
3
4
HOURS INCUBATION
TIME OF HEATING (HOURS)

fate of this DNA following incubation of the cells for a time period sufficient to allow uptake and integration of DNA was determined (Table 6). During the 2 h incubation period following the termination of DNase-resistant DNA-binding both cell types released similar amounts of radioactivity, 95% of which was soluble in cold 8% trichloroacetic acid. Approximately 17% of the radioactivity bound to competent cells was transported into the cell cytoplasm but there was essentially no [^{32}P]DNA uptake in 42°C-treated cells. The very small amount of radioactivity which appeared in the cytosol fraction of 42°C-treated cells was quite possibly washed from the spheroplast membranes when they were removed by centrifugation. A greater proportion of the [^{32}P]DNA remained bound to the 42°C-treated cell envelope consistent with a failure to transport DNA into these cells. All of the [^{32}P]-labeled material in the cytosol of transformed cells was ethanol-precipitable. The chromosomal and [^{32}P]DNA in osmotically lysed spheroplasts migrated as a tight band (molecular weight > 1.7 x 10^7) during electrophoresis in agarose (Appendix 5). These results were consistent with the hypothesis that 42°C-treated cells were unable to transport DNA across the cell envelope.

DNA binding to competent and noncompetent cells

It could not be determined whether cells which were competent prior to 42°C-treatment were the same cells which reacquired competence during subsequent incubation. Competent *A. vinelandii* which were exposed to saturating strain 113 (Rif^r) DNA prior to 42°C treatment were not transformed to streptomycin-resistance with strain 114 DNA at any time during the 8 h post-heating incubation at 30°C. Exposure of 42°C-treated cells to saturating Rif^r DNA prevented subsequent transformation by Str^r DNA and Rif^r transformants were not detected during the 8 h incubation. Competent cells exposed to excess strain 113 DNA also could not be transformed subsequently with strain 114 DNA even 8 h after the first exposure to strain 113 DNA.

In each of the three cases outlined above, preexposure of cells to excess purified DNA did not prevent these cells from binding a second [^{32}P]DNA in a DNase-resistant state. Approximately equivalent amounts of [^{32}P]DNA were bound after the first and second exposure of competent cells to DNA. Precompetent strain UW1 grown in Fe-limited Burk medium for 4, 8, and 12 h (Page and von Tigerstrom, 1979) bound as much [^{32}P]DNA in a DNase-resistant state (average: 1.2×10^5 cpm/10^8 cells) as the competent population which was present after 16 and 24 h incubation. [^{32}P]DNA also was bound in a DNase-resistant state by noncompetent cells (Page and von Tigerstrom, 1979) prepared by growth in Fe-sufficient Burk medium (9.1×10^4 cpm/10^8 cells) or in Fe-limited Burk medium containing glutamate as the sole N-source (5.9×10^5 cpm/10^8 cells). The ability of *A. vinelandii* to bind DNA in a DNase-resistant state was, therefore, not a competence-specific characteristic. DNase-resistant DNA binding was dependent on magnesium ions. Competent cells which had been washed free of excess magnesium ions and exposed to DNA in the absence of added magnesium ions bound less than 1.0×10^2 cpm of [^{32}P]DNA/10^8 cells and no transformants were generated.

TABLE 6. Binding and uptake of [^{32}P]DNA[a]

Recipient[b]	DNase-resistant ^{32}P bound		^{32}P released during 2 h incubation		^{32}P released upon spheroplast formation		^{32}P bound to spheroplast membranes		^{32}P in cell cytosol	
	cpm[c]	%	cpm	%	cpm	%	cpm	%	cpm	%
competent cells	3280(±215)[d]	100	197(±35)	6.0	2309(±473)	70.4	212(±29)	6.4	562(±120)	17.2
42°C-treated cells	2945(±510)	100	136(±18)	4.6	2002(±128)	68.0	780(±23)	26.5	26.7(±3.0)	0.95

[a] Procedure is described in Experimental Procedures.

[b] Competent cells were transformed to Nif$^+$ at a frequency of 4.9×10^{-2}. No competent cells were detected in the 42°C-treated culture.

[c] Measurements of radioactivity are counts per min per 10^8 cells.

[d] Standard deviation.

Heterologous DNA competition

Competent strain UW1 was transformed to Nif$^+$ at high frequency with saturating DNA from *A. vinelandii* strains 113 (frequency, 1.0×10^{-2}), 114 (frequency, 2.5×10^{-2}) or UW (frequency, 8.4×10^{-3}). Transformation with saturating amounts of DNA prepared from *A. chroococcum* and *A. paspali* resulted in frequencies of 4.0×10^{-3} and 2.2×10^{-3}, respectively. Strain UW1 was transformed at very low frequency by saturating DNA from *A. beijerinckii* (frequency, $3.2 \times 10\text{-}5$), *B. indica* (frequency, 1.5×10^{-6}), *R. meliloti* (frequency, 1.4×10^{-6}) and *R. trifolii* (frequency, 3.3×10^{-6}). Strain UW1 was not transformed by DNA isolated from the distantly related bacteria *A. insignis* and *A. macrocytogenes* or the unrelated bacteria *K. pneumoniae* and *C. pasteurianum*.

Several of the homologous and heterologous DNA species were partially purified and tested for their ability to compete with strain 113 DNA. Competing DNA was mixed with an equivalent amount (1.0 μg/10^8 cells) of strain 113 DNA and the frequency of Rif$^+$ marker transformation was assayed. Nontransforming *A. macrocytogenes* DNA and very poorly transforming *B. indica* DNA decreased the Rif$^+$ transformation frequency by 50-52%. The ability of these two DNA species to block transformation was not altered significantly when recipient cells were preexposed to the heterologous DNA 20 min prior to strain 113 DNA. DNA prepared from *A. beijerinckii*, *A. paspali* and *A. vinelandii* strain 114 similarly decreased transformation by strain 113 DNA by 55, 59, and 67%, respectively. Although the ability of these related DNA species to compete with strain 113 DNA did not correlate precisely with their ability to transform UW1, the relative order was the same. These DNA species blocked transformation more efficiently when cells were preexposed to blocking DNA prior to the addition of strain 113 DNA and the relative order of blocking ability remained unchanged (Appendix 6). Treatment of cells with DNase after exposure to heterologous or homologous DNA did not alter the blocking ability to the competing DNA species. Blocking by these DNA species was dependent on magnesium ions.

Bacteriophage ØW-14 DNA bound in a DNase-resistant state to competent *A. vinelandii* blocked transformation by an equivalent amount (1.1 μg) of strain 113 homologous DNA more effectively than any other heterologous DNA including heterospecific DNA species. Under these conditions ØW-14 DNA produced a 64% decrease in the Rif^+ transformation frequency. The ability of ØW-14 to block transformation by homologous DNA was concentration dependent such that transformation of strain UW1 was completely blocked by exposing 10^8 cells to greater than 5 μg of ØW-14 DNA.

Discussion

Genetic transformation of *A. vinelandii* was notably similar to that of *H. influenzae*. DNase-resistant DNA binding to both organisms demonstrated a brief lag (from 1-3 sec), was saturated after 5-10 min exposure to excess DNA (Deich and Smith, 1980; Stuy and Stern, 1964), and occurred external to the cytoplasmic membrane (Deich and Hoyer, 1982; Kahn et al., 1982). In both transformation systems, DNA uptake was not accompanied by the release of an equivalent amount of acid-soluble deoxyribonucleotides from the cell surface (Stuy and Van der Have, 1971). Therefore a nuclease such as that active in Gram-positive transformation systems (Davidoff-Abelson and Dubnau, 1973; Lacks et al., 1974; Mulder and Venema, 1982) which generates single-stranded DNA during uptake does not have an equivalent role in *A. vinelandii* transformation. Single-stranded DNA probably is not an acceptable donor in this system because heat-denatured *A. vinelandii* DNA (60 μg DNA/ml SSC at 100°C for 25 min) is 1200-fold less active in transformation than native DNA (Appendix 7). Since biologically active donor DNA was recovered from transformed *A. vinelandii* as early as 1 min after DNA addition it is likely that the donor DNA is transported in a double-stranded form typical of Gram-negative transformation systems (Barnhart and Herriott, 1963; Biswas and Sparling, 1981; Notani and Goodgal, 1966; Stuy, 1965).

In contrast to other heat-sensitive transformation systems (Deddish and Ravin, 1974; McCarthy and Nester, 1969; Ravin and Ma, 1972) the major effect of heating competent *A. vinelandii* was limited to a single time period prior to the completion of DNase-resistant DNA binding. Despite this, heat-treated formerly competent cells were not impaired in their ability to bind DNA in a DNase-resistant state. The small, but consistently observed, enhancement of transformability at 37°C that occurred concurrently with the completion of DNase-resistant DNA binding indicated that DNase-resistant DNA binding and the heat-sensitive step were closely linked temporally. DNase-resistant DNA binding clearly preceded the heat-sensitive step in the transformation process. The significant observation was that little or no radioactivity and no biologically active donor DNA marker was recovered from the cytoplasm of 42°C-treated cells which had bound saturating amounts of [^{32}P]DNA in a DNase-resistant state. The data support the hypothesis that the heat-sensitive event was involved in DNA transport across the cell envelope. The existence of a DNA uptake protein (Concino and Goodgal, 1981; Sutrina and Scocca, 1979) which is particularly heat-labile may explain the need for protein synthesis during competence recovery. The nature of the heat-sensitive event in *A. vinelandii* transformation may be unique. In other heat-sensitive transformation systems, the loss of transformability is almost totally attributable to a loss of the ability to bind DNA on the cell surface in a DNase-resistant state (Barnhart and Herriott, 1963; Deddish and Ravin, 1974; McCarthy and Nester, 1969; Ravin and Ma, 1972).

Similar to *Neisseria gonorrhoeae* (Dougherty et al., 1979), *A. vinelandii* exposed to homologous DNA were unable to be transformed by a second DNA species. A surprising observation was that these cells were capable of further DNase-resistant DNA binding. These results suggest that there may be two types of DNA binding to competent *A. vinelandii* both of which convey resistance to DNase. DNA receptors of type 1 are proposed to be responsible for the possibly less specific form of DNase-resistant DNA binding, which may be preliminary to DNA binding for transformation, but which occurs in the absence of transformation. Type 2 receptors are proposed to be those which bind transforming DNA during the initial stages of the transformation process. These two DNA receptor types are therefore somewhat analogous to those described in *N. gonorrhoeae* (Dougherty et al., 1979). Both types of DNA binding receptors are heat-stable as 42°C-treated cells participated in both types of DNase-resistant DNA binding. Furthermore, 42°C-treated cells failed to transport DNA indicating that both types of receptors must be located in the cell envelope. The existence of type 1 receptors may explain the phenomenon of DNase-resistant DNA binding to non-competent cells, a characteristic apparently unique to *A. vinelandii*. Obviously, it cannot be determined whether noncompetent cells a possess type 2 receptor until it has been physically identified.

Competent *A. vinelandii* discriminate against transformation by heterologous DNA, a characteristic unique to Gram-negative organisms which are naturally grown to competence (Carlson et al., 1983; Danner et al., 1980; Dougherty et al., 1979; Grave et al., 1982; Scocca et al., 1974; Sisco and Smith, 1979). A possible exception to this trend was the anomolous behavior of ØW-14 DNA, a DNA species which also is exceptionally competitive in the *B. subtilis* transformation system (Lopez et al., 1980; 1982). When competent *A. vinelandii* are generated by the artificial system used to produce competent *Escherichia coli* (Cohen et al., 1972), however, they show the same lack of discrimination against heterologous DNA as does *E. coli* (Brown et al., 1981; David et al., 1981).

Competent *A. vinelandii* demonstrated a certain lack of discrimination against binding heterologous DNA in a DNase-resistant state which may be explained by the existence of type 1 receptors. The ability of heterologous nontransforming DNA to inhibit transformation by homologous DNA was proportional to the concentration of the two competing DNA species but was not enhanced by the prior exposure of cells to blocking

DNA unlike the case with competing transforming DNA. These results suggest that DNA binding to type 1 receptors was preliminary to binding to type 2 receptors or that DNA binding to type 1 receptor sterically hindered transforming DNA binding to type 2 receptor.

It is possible that type 2 receptors were responsible for discrimination against heterologous DNA other than 0W-14 DNA by either preventing or limiting its uptake. Thus, type 2 receptors would resemble the surface receptors of competent *H. influenzae* (Deich and Hoyer, 1982; Deich and Smith, 1980; Kahn et al., 1982) and *N. gonorrhoeae* (Dougherty et al., 1979) which recognize specific uptake sequences on transforming DNA. These sequence specific receptors are reputed to bind and transport one molecule of DNA each but *A. vinelandii* doubly transformed by unlinked markers is often observed (Doran and Page, unpublished data) suggesting that there are several type 2 receptors per competent cell. The lag before the loss of transformation competence during 37°C treatment suggests that there are also several copies of heat-labile DNA uptake site per competent cell.

No transformants were generated from 42°C-treated cells which were incubated for 8 h following heating and exposure to homologous DNA. This suggests that DNA uptake may have to occur concomitant with DNA binding to type 2 receptors. The rapid recovery of donor DNA marker (*nif*) from competent cells demonstrated that DNA transport was occurring very soon after DNase-resistant DNA binding. DNA binding to type 2 receptor may alter the receptor preventing it from becoming properly associated with the DNA uptake mechanism at a later time. Type 2 receptors, therefore, may be synthesized only once in the competence cycle or if they are synthesized during competence recovery they may be inactivated by DNA prebound to the cell.

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Note Added in Proof

Strain UW1 was not transformed to antibiotic resistance with the common plasmid cloning vectors pBR 322 (4. kb, tet^{r}) or its derivative pAT 153 or with pACYC 184 (4.0 kb, Cam^{r}). Several larger plasmids prepared from *E. coli* (Appendix 8) including 1010 (5.5 kb, Str^{r}), d53-R1-307 (33 kb, tet^{r}), J53-R1 (62 kb, cam^{r}), DT-833 (112 kb, tet^{r}) and DT-831 (166 kb, tet^{r} kan^{r}) also failed to transform strain UW1. Strain UW91 was not transformed to Nif^{+} with pSA30 (W. Page, unpublished data) which carried the structural genes for *K. pneumoniae* nitrogenase. In these instances the integration of transforming DNA would not have been necessary for the generation of transformants. The results, therefore, may support the hypothesis that discrimination against heterologous DNA by competent occurred prior to DNA uptake.

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V. A Possible Role for Adhesion Sites in DNA Uptake by Competent *Azotobacter vinelandii*

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Summary

Adhesion sites between the cytoplasmic and outer membrane were revealed in competent cells by freeze-etch electron microscopy as localized plateaus of outer membrane material adherent to the cytoplasmic membrane. These adhesion sites were absent from 42°C-treated, formerly-competent cells, which failed to transport transforming DNA across the cell envelope, but appeared concurrently with competence recovery at 30°C. Consistent with the disruption of these adhesion sites at 42°C was the copurification of three inner membrane proteins with outer membrane material following heat treatment of competent cells. Noncompetent cells prepared by growth in Burk medium or modified competence-induction medium containing glutamate as sole nitrogen source did not possess adhesion sites demonstrable by freeze-etch techniques. Adhesion sites viewed in thin sections of competent cells were not significantly reduced following 42°C-treatment indicating that most adhesion sites were not as temperature-sensitive as those which

correlated with transformation competence. The results suggest that DNA uptake by *A. vinelandii* may occur at competence-specific, heat-labile adhesion sites.

Introduction

Competence for genetic transformation of *Azotobacter vinelandii* decays rapidly at 37 to 42°C (Page and von Tigerstrom, 1979). Heat-treated, formerly-competent cells bind normal amounts of [^{32}P]DNA in a DNase-resistant state but no radioactivity or biologically-active donor marker can be isolated from the cytoplasm. The survival and expression of donor marker following uptake is relatively insensitive to heating at 42°C. Therefore DNA transport across the cell envelope appears to be the single heat-sensitive event in *A. vinelandii* transformation (Chapter IV). The results of studies described here suggest that the disruption of specific adhesion sites at 42°C may account for the lack of DNA uptake by 42°C-treated cells.

Adhesion sites have been observed in Gram-negative bacteria in thin sections of plasmolysed cells (Bayer, 1968) and by freeze-etch electron microscopy (Bayer and Leive, 1977). Various functions attributed to adhesion sites include the export of newly synthesized lipopolysaccharide (Bayer and Leive, 1977; Bayer et al., 1982; Muhlradt and Golecki, 1975), capsular polysaccharide (Bayer and Thurow, 1977) and outer membrane proteins (Smit and Nikaido, 1978). Adhesion sites are also the sites of adsorption of several bacteriophage (Bayer, 1979). The recent observation that surface DNA binding sites on competent *Haemophilus influenzae* are located adjacent to adhesion sites led to the suggestion of a possible involvement of adhesion sites in the uptake of transforming DNA (Kahn et al., 1982). Similarly, DNA binding sites on competent *Streptococcus pneumoniae* appear to be located at wall-membrane junctions analogous to the adhesion sites of Gram-negative bacteria (Seto et al., 1975).

(Preliminary results of this study were presented at the XIIIth Meeting of the International Congress of Microbiology, 8-13 August 1982, Boston, Mass.)

Experimental procedures

Bacterial strains

The transformation recipient strain *Azotobacter vinelandii* OP strain UW1 (Nif^- $capsule^-$, (Bishop and Brill, 1977)) was grown on Burk medium containing 1.1 g of ammonium acetate per liter (Page and Sudoff, 1976). The source of Nif^+ donor DNA, *A. vinelandii* ATCC 12837 strain 113, was grown on nitrogen-free Burk medium. Liquid cultures were incubated at 30°C and 170 rpm in a water bath gyrotory shaker model G-76 (New Brunswick Scientific Co., New Brunswick, N.J.). All cultures were incubated at 30°C.

Transformation

Competent *A. vinelandii* were prepared by 20 to 24 h of growth in iron-limited Burk medium (Page and von Tigerstrom, 1978). Crude lysate DNA was prepared from strain 113 by lysis of cells in 15 mM saline-15 mM sodium citrate, pH 7.0, containing 0.05% sodium dodecyl sulfate

(SDS) by heating at 60°C for 60 min (Page and Sadoff, 1976). Competent strain UW1 cells were transformed at 30°C in Burk buffer, pH 7.2, containing 8 mM $MgSO_4$ (Page and von Tigerstrom, 1979), using excess crude lysate DNA (10 μg DNA/10^8 cells). DNase-resistant DNA binding was terminated after 20 min by addition of DNase 1 (final concentration 4 μg/ml). Nif^+ transformants were detected by plating cells on nitrogen-free Burk medium. Transformation frequency was calculated as the number of Nif^+ transformants detected per number of viable cells plated on selective medium. All determinations were made at least twice.

Membrane isolation

Membrane vesicles prepared by disruption of cells in a French pressure cell were isolated from sucrose gradients described previously (Page and von Tigerstrom, 1982) following low speed centrifugation (5000 rpm, 20 min) of the cell lysate to remove unbroken cells and granules of poly-β-hydroxybutyrate. Gradient fractions of 0.4 ml were collected from the bottom of the gradient and sucrose concentration was determined by measuring refractivity. NADH oxidase activity was determined as described by Osborn et al. (1972). The pooled peak fractions were assayed for protein (Lowry et al., 1951) and lipopolysaccharide (Osborn et al., 1972) using bovine serum albumin and 2-ketodeoxyoctonate, respectively, as standards. Poly-β-hydroxybutyrate concentration was determined by the method of Law and Slepecky (1961).

Electron microscopy

Cells for freeze-etch electron microscopy were pelleted from culture, washed once with 1 volume of 5 mM potassium phosphate buffer, pH 7.2, and resuspended in a minimum volume of the same. A droplet of thick cell suspension on a 3 mm gold disc was frozen in Freon 22 and stored under liquid N_2 as described by Moor (1969). The frozen specimens were fractured and then etched for 30 s as described by De Voe et al. (1971) using a Balzers BA 360M apparatus. Platinum-carbon replicas were mounted on Formvar-coated 200 mesh copper grids.

Thin-sections of plasmolyzed cells were prepared by a modification of the method of Bayer and Thurow (1977). Cells plasmolyzed by 5 min incubation in iron-limited Burk buffer, pH 7.2, containing 20% w/v sucrose were fixed for 1 h at room temperature by suspending in 0.2 volume of Kellenberger buffer (Kellenberger et al., 1958) without calcium, pH 7.0, containing 20% w/v sucrose and 2% formaldehyde made from paraformaldehyde. After 30 min fixation the pH was readjusted to 7.0 using NaOH. The cells were centrifuged and the pellet was gently resuspended in Kellenberger buffer, pH 7.0, containing 20% (w/v) sucrose, 10% tryptone and 1% (w/v) OsO_4 and held at room temperature for 18 h. Fixed cells were embedded in 2% Noble agar and thin sections of Epon embedded specimens were prepared as described previously (Page, 1982).

Membrane fragments isolated from sucrose gradients were washed once in 8 mM Tris buffer, pH 7.2, by centrifugation (30,000 rpm, 60 min) prior to negative staining (Horne, 1961) on Formvar-coated 200 mesh, copper grids.

All specimens were examined in a Phillips 300 electron microscope.

Electrophoresis

Samples containing membrane fractions were solubilized in 0.5 volume of sample buffer containing 0.187 M Tris-(hydroxymethyl) aminomethane (Tris), pH 6.8, 6% sodium dodecyl sulfate, 15% β-mercaptoethanol and .006% bromophenol blue prior to application to the gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 1.5 mm thick acrylamide slab gels formed in a Hoeffer model 220 electrophoresis cell followed a modification of the procedure of Laemmli (1970) described previously (Page and von Tigerstrom, 1982). Proteins were stained with Coomassie blue R250 in isopropanol and acetic acid as described by Fairbanks et al. (1971).

Chemicals

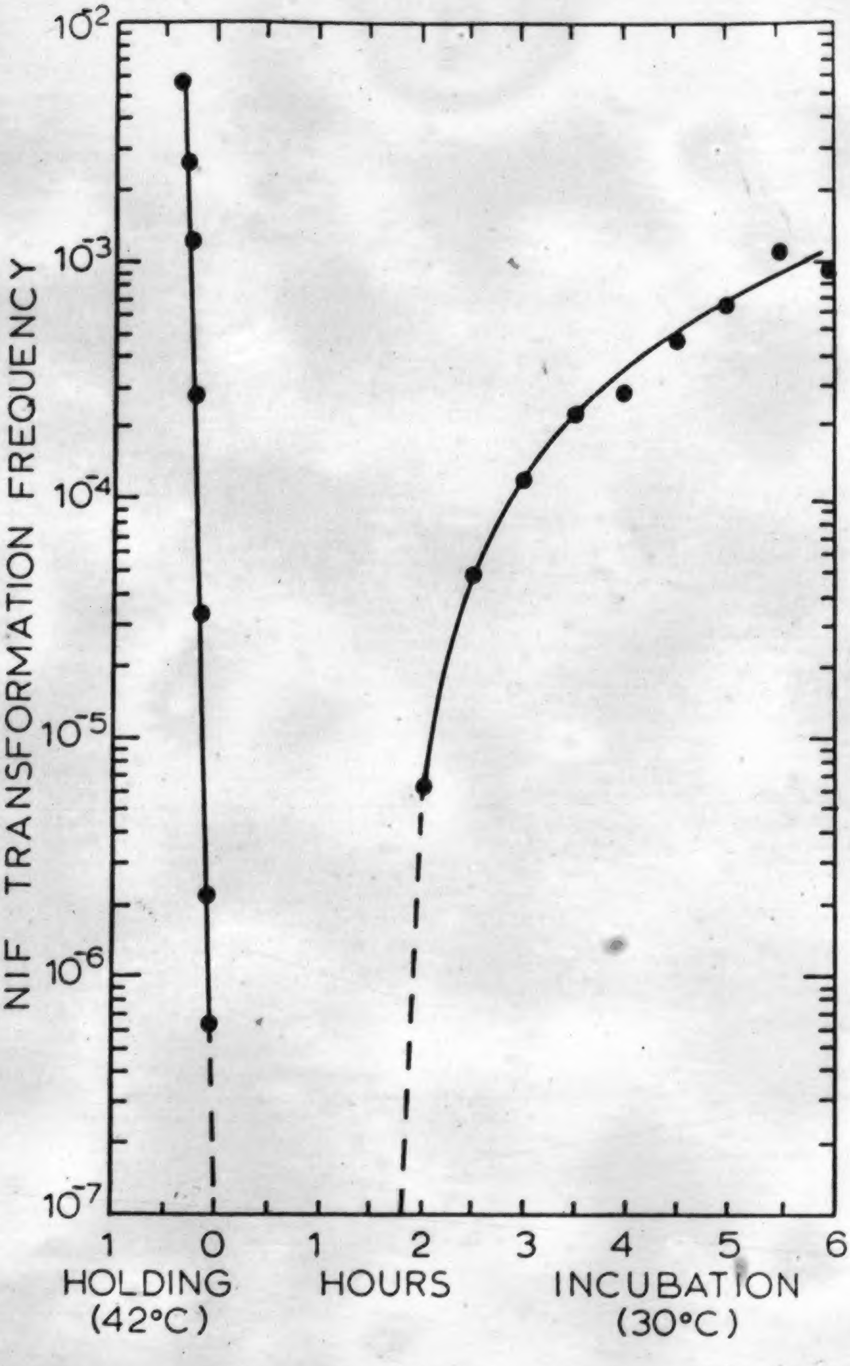
Electrophoresis grade acrylamide and N,N'-methylene bisacrylamide were purchased from Eastman Kodak Co. Other chemicals for electrophoresis were obtained from Bio-Rad Laboratories (Mississauga, Ontario). All other fine chemicals were reagent grade and most were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Results

Freeze-etch studies of competent and noncompetent cells

Competent cells in a population of strain UW1 exhibited an exponential loss of competence at 42°C which was complete after 20 min (Fig. 10). The competence of the culture redeveloped during subsequent incubation at 30°C.

Figure 10. Loss and recovery of competence. A competent culture of strain UW1 was held at 42°C for 30 min and at time intervals aliquots of culture were equilibrated to 30°C and competence was assayed. The culture was then incubated at 30°C and at time intervals the level of competence recovery was determined.


NIF TRANSFORMATION FREQUENCY
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
1
0
1
2
3
4
5
6
HOLDING (42°C)
HOURS
INCUBATION (30°C)

Cells were examined by freeze-etch electron microscopy to determine whether 42°C-heating induced observable changes in membrane organization. These studies revealed that cleavage planes through the inner membrane of competent cells were often interrupted by "plateaus" of outer membrane material on which the outer membrane fracture face was usually exposed (Fig. 11a). Similar structures observed in *Escherichia coli* were considered to represent adhesion sites (Bayer and Leive, 1977). Approximately 25% of cells in a competent population (Nif$^+$ transformation frequency 1.6×10^{-2}) which had fractured through the inner membrane exhibited this feature and often 3 to 9 plateaus were present per cell. An accurate number of plateaus per cell could not be estimated as a fracture plane which had switched to the hydrophobic region of the outer membrane often failed to return immediately to the inner membrane fracture plane possibly preventing similar structures from being observed. The 42°C-treated formerly-competent cells did not possess plateaus demonstrable by freeze-etch techniques (Fig. 11b) but plateaus were present in cells following competence redevelopment (Nif$^+$ transformation frequency 1.5×10^{-2}, Fig. 11c). Freeze-etched preparations of non-competent strain UW1 prepared by growth in iron-sufficient Burk medium or modified iron-limited competence induction medium containing glutamate as sole N-source (Page and von Tigerstrom, 1978) did not possess these plateaus (Fig. 12). Plateaus were apparent in freeze-etched preparations of strain UW1 cells after 13 h incubation in competence induction medium just prior to the appearance of competence (Page and von Tigerstrom, 1978). These plateaus continued to be prevalent in cells following 36 h incubation

Figure 11. Freeze-etch replicas of competent and 42°C-treated, formerly competent, strain UW1. Samples were prepared from a portion of a competent culture prior to 42°C-treatment (a), following 42°C-treatment (b), and from a portion of the 42°C-treated culture following competence recovery (c). The symbols are those recommended by Bayer and Leive (1977): convex fracture face, ⌒; outer surface of outer membrane, OMO; fracture face of outer membrane, OMF; outer face of inner membrane, IMO; fracture face of inner membrane, IMF; plateaus of outer membrane on inner membrane cleavage plane, PL-OMF; regularly arrayed protein surface layer (W.H. Bingle, J.L. Doran, and W.J. Page, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K155, p. 202), rs. ⌀ indicated the direction of platinum shadowing. Bar, 0.5 μm.

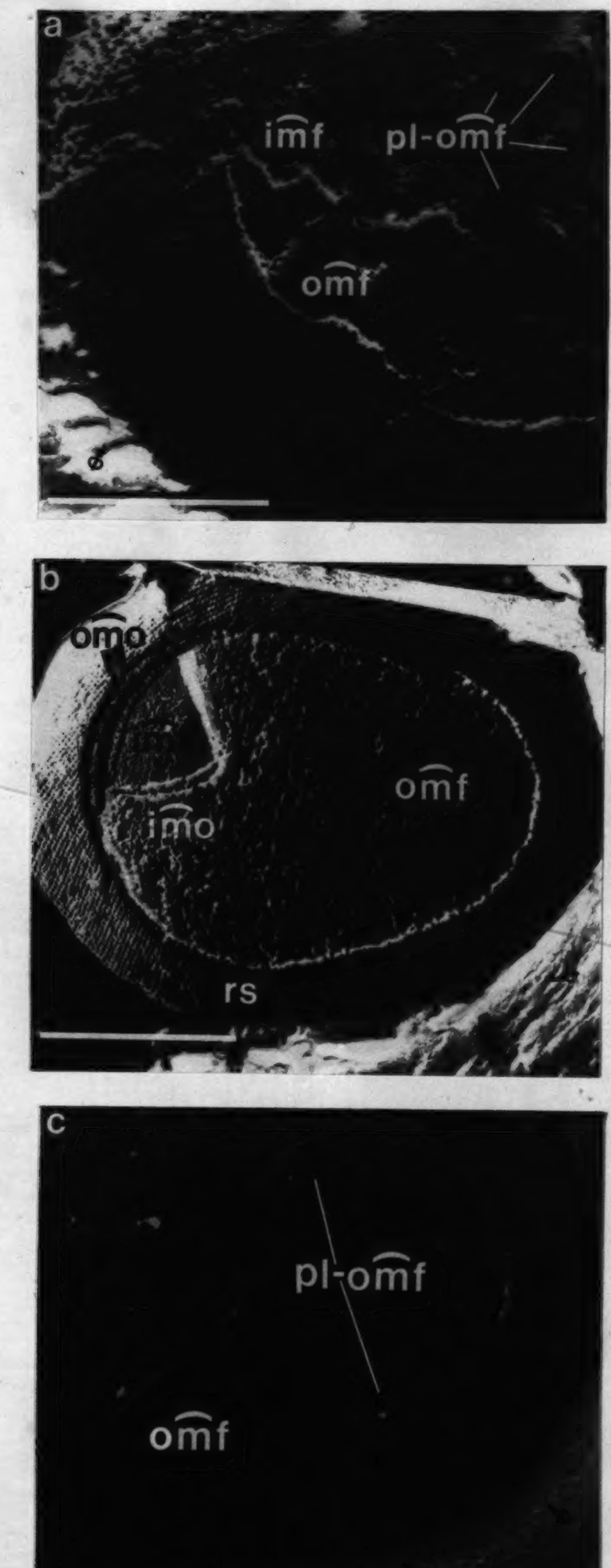
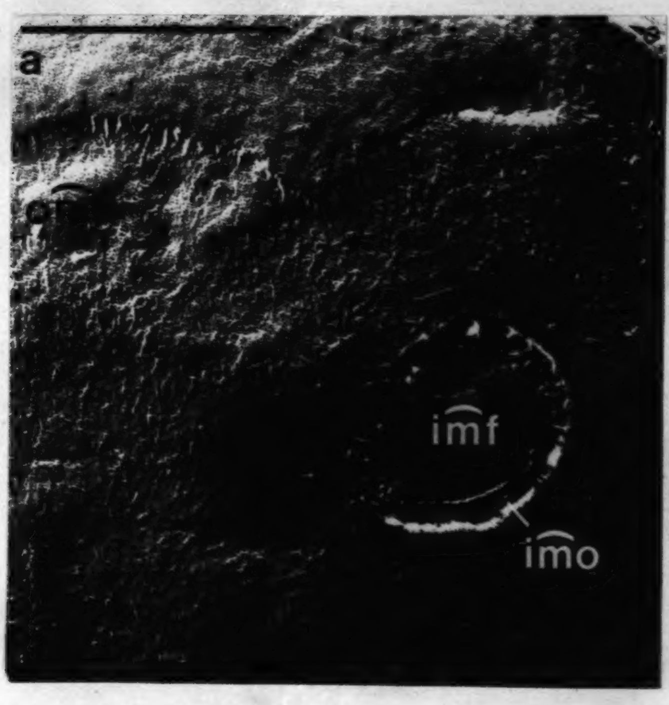
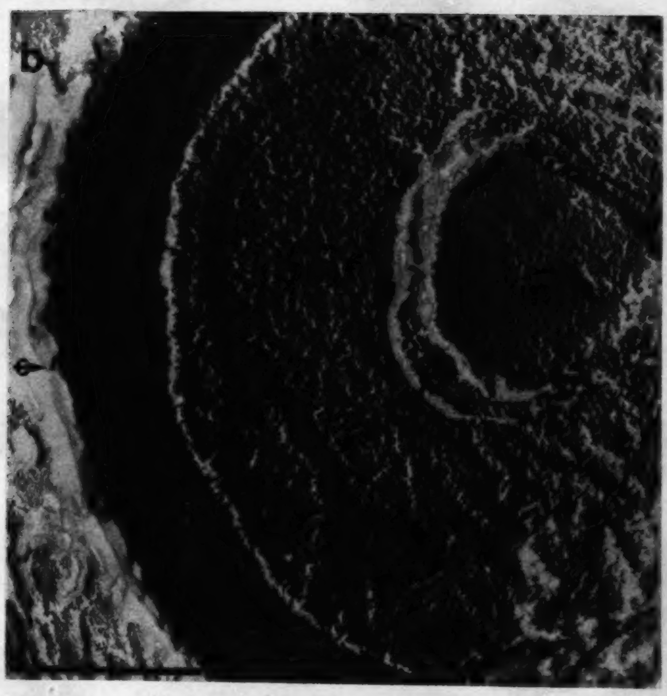

a
imf
pl-omf
omf
e
b
omo
omf
imo
rs
c
pl-omf
omf

Figure 12. Freeze-etch replicas of noncompetent strain UW1 prepared by growth in Burk medium (a) and iron-limited Burk medium containing glutamate as sole nitrogen source (b). The symbols were defined in the legend of Figure 11.


a
om
imf
imo


b

in competence induction medium although competence had declined drastically (Nif+ transformation frequency 1.3 x 10^{-6}). The normal decline in competence after extended incubation of cells in iron-limited Burk medium (Page and von Tigerstrom, 1978), however, may be caused by prolonged O_2 limitation (Page, 1982) rather than a specific loss of competence-related function.

Adhesion sites (Bayer's junctions) observed in thin-sections of plasmolysed competent strain UW1 appeared as two structural types (Fig. 13) very similar to those described in *E. coli* (Bayer, 1968; Bayer and Starkey, 1972). Most adhesion sites observed were of the type where the inner and outer membranes were closely apposed but rarely adhesion sites were apparent as duct-like structures. There was not a significant reduction (P = 0.05) in the number of adhesion sites viewed in thin sections of competent cells following 42°C treatment indicating that most adhesion sites are not as heat labile as the membrane plateaus observed in freeze-etch preparations.

There was an average of 1.5 adhesion sites in the plane of thin sections of randomly oriented, completely plasmolysed cells. Given that the average thickness of these silver-grey sections was approximately 600-800Å (Peachy, 1958) and that the cells had an average diameter of 1.5 μm a minimal qualitative estimate of adhesion sites per cell was 30 to 40. This was considered a minimal estimate because sections of completely plasmolyzed cells usually contained fewer adhesion sites than thin sections through partially plasmolyzed cells indicating the fragile nature of these structures (Bayer and Starkey, 1972).

Figure 13. Electron micrograph of a thin section from a plasmolyzed cell from a competent population of strain UW1. The specimen was stained with 5% uranyl acetate in methanol and 0.4% lead citrate in 0.1 N NaOH. Adhesion sites were visible as regions where the inner and outer membranes remained closely apposed (small arrows) or as duct-like structures (large arrow). A granual of poly-β-hydroxybutyrate (PHB) occupied the center of the section. Bar, 0.5 μm.

PHB

Membrane fractions from competent and 42°C-treated cells

Membrane fractions isolated from sucrose gradients with a density intermediate between inner membrane and outer membrane fractions have been suggested to be enriched for adhesion sites (Bayer et al., 1982; Tomita et al., 1976). Comparison of the sucrose gradient profiles of membrane material from competent and 42°C-treated cells (Fig. 14) suggested the 42°C-treatment resulted in a small reduction in the amount of intermediate density material. This was misleading as measurements of protein concentration of pooled peak fractions from competent (0.25 mg protein/ml) and 42°C-treated cells (0.37 mg/ml) indicated no such change. The differences in peak shapes probably reflected differences in the amounts of residual contaminating poly-β-hydroxybutyrate (PHB) in the intermediate density fraction from competent (0.37 mg PHB/mg protein) and 42°C-treated cells (0.059 mg PHB/mg protein). Examination of membrane preparations by phase-contrast light microscopy and electron microscopy of negatively stained preparations (Fig. 15) revealed numerous poly-β-hydroxybutyrate granules in the intermediate-density preparation, as expected (Page and von Tigerstrom, 1982), and a lesser amount in the outer membrane peak 2 fraction some of which appeared to be broken into strands. The existence of poly-β-hydroxybutyrate material of varying density was previously reported (Nickerson, 1982).

There was generally more material in the lysates of 42°C-treated cells (Fig. 14) including approximately two-fold more protein and poly-β-hydroxybutyrate in the outer membrane fractions. The reason for this is unclear but the effect was not reproduced by treating French

Figure 14. Separation of membrane fragments on sucrose gradients. A competent culture of strain UW1 (Nif$^+$ transformation frequency 1.8×10^{-2}) was portioned into 2 equal volumes one of which was heated at 42°C for 30 min to destroy competence. Membrane vesicles prepared from (a) competent and (b) 42°C-treated cells by French pressure cell disruption were isolated on sucrose gradients. Fractions were assayed for absorbance at 280 nm (○), percent sucrose (△) and NADH oxidase activity (□). The peak fractions designated (A) outer membrane peak 1 (OM1), (B) outer membrane peak 2 (OM2), (C) intermediate-density material (ID) and (D) inner membrane fragments (IM) were pooled for further analysis.

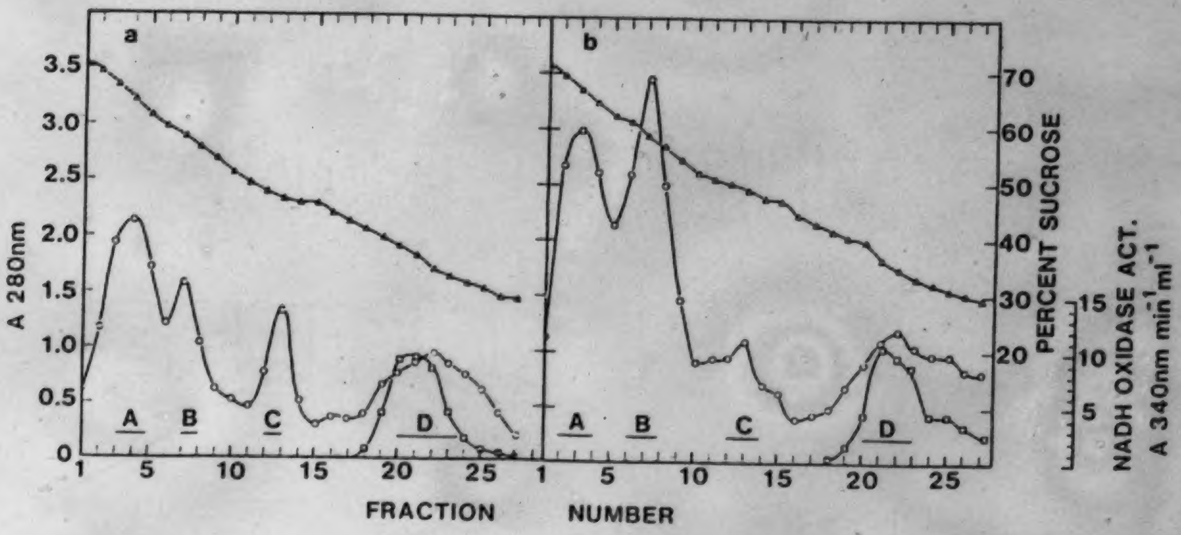
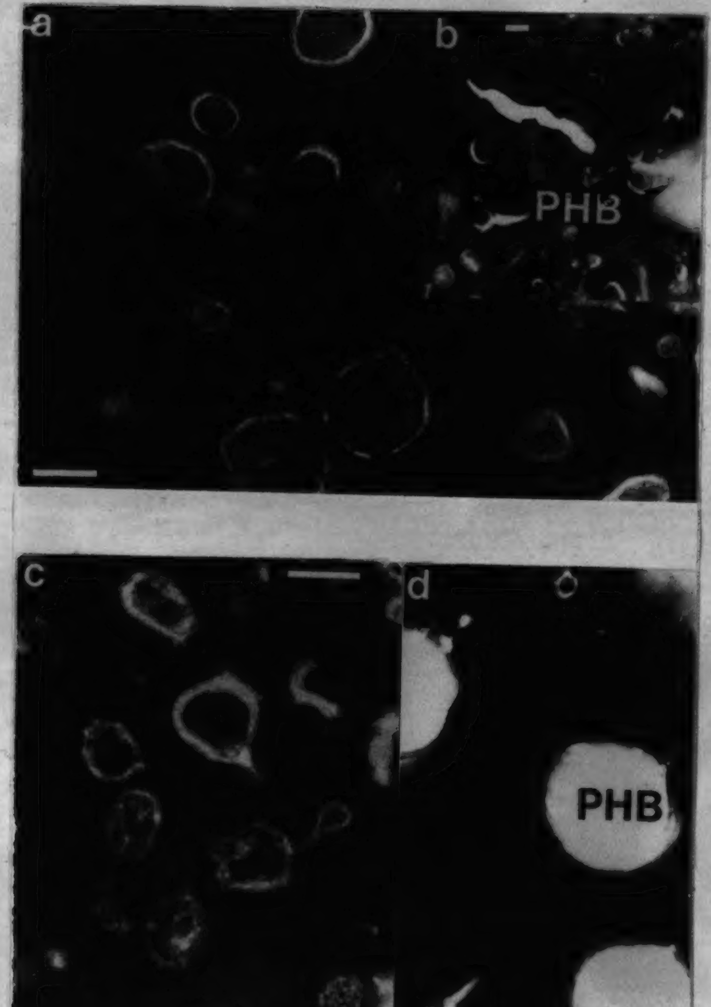

a
b
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
A 280nm
70
60
50
40
30
20
PERCENT SUCROSE
15
10
5
NADH OXIDASE ACT.
A 340nm min^{-1}ml^{-1}
A
B
C
D
1
5
10
15
20
25
FRACTION
NUMBER

Figure 15. Membrane fragments, from various sucrose gradient fractions, negatively stained in 3% phosphotungstate: (a) membrane fragments from outer membrane peak 2 (OM2); (b) granule and strand of poly-β-hydroxybutyrate present in OM2 fraction; (c) membrane fragments and (d) granules of poly-β-hydroxybutyrate from the intermediate density fraction; (e) membrane fragments from the inner membrane fraction. Bar, 0.1 μm.

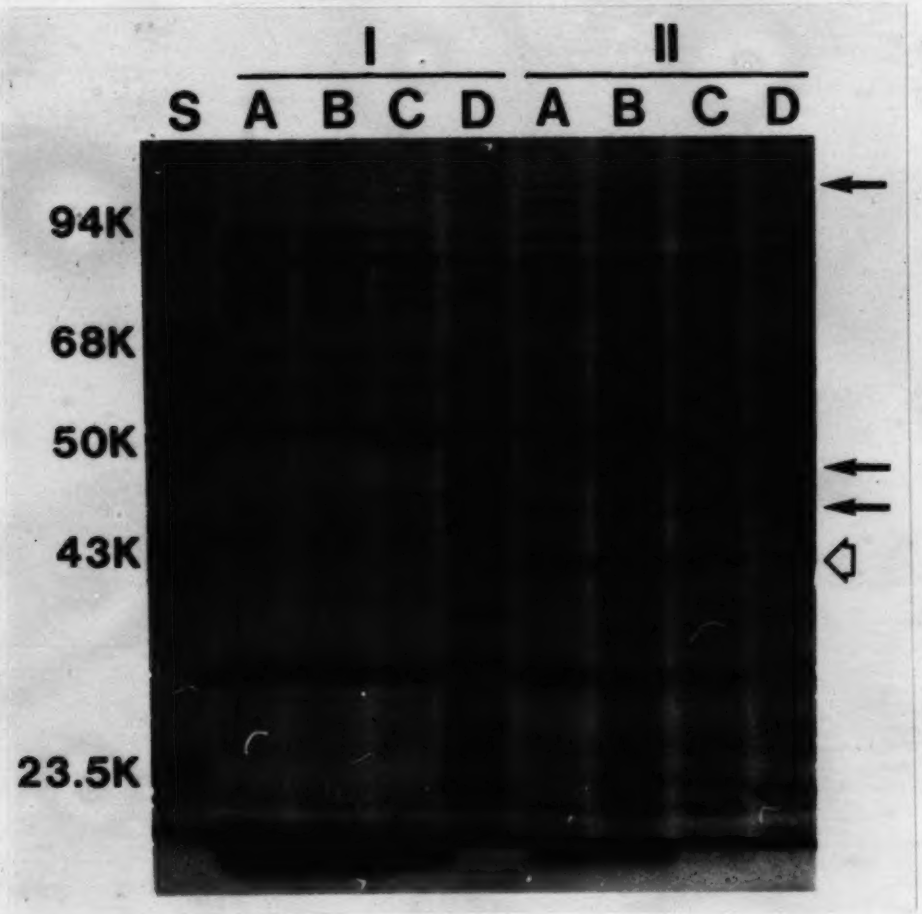

a
b
PHB
c
d
PHB
e

pressure cell lysate of unheated cells at 42°C prior to density gradient centrifugation and may therefore reflect efficient breakage of 42°C-treated cells during French pressure cell disruption.

The sucrose gradient peak fractions indicated in Fig. 14 were analyzed by SDS-PAGE (Fig. 16). The protein content of the intermediate density membrane material of competent cells was identical to that of outer membrane fragments with the exception of a protein of apparent molecular weight 49,000 which was unique to the intermediate density fraction. The density of this fraction was reflected in the ratio of lipopolysaccharide to protein (8.8 μg/mg protein) in this fraction relative to the other major fractions (OM1-38, OM2-22, IM-5.1 μg/mg protein). Although some inner membrane bilayer might be expected to be present in the intermediate density fraction (Hancock and Nikaido, 1978), no NADH oxidase activity (Fig. 14) or inner membrane proteins (Fig. 16) were detected. The similar high-contrast profile of negatively stained inner and outer membrane fragments (Fig. 15) prevented visual estimation of the contribution of each to the intermediate density fraction.

A surprising observation was that the intermediate-density and outer membrane preparations from 42-treated cells possessed three proteins of apparent-molecular-weights 44,500, 47,000 and approximately 106,000 (Fig. 16, closed arrows) which purified as inner membrane proteins in unheated cells. Since no NADH oxidase activity or other inner membrane proteins were detected, this did not represent simple contamination of this fraction with the inner membrane. This could be explained by the temperature induced disruption of adhesion sites which

Figure 16. SDS-PAGE of membrane fragments from competent and 42°C-treated cells. The pooled peak sucrose gradient fractions (Fig. 5) designated (A) outer membrane peak 1, (B) outer membrane peak 2, (C) intermediate density membranes and (D) inner membrane material from (I) competent cells and (II) 42°C-treated formerly-competent cells were analyzed by SDS-PAGE. Membrane proteins were solubilized by freezing and thawing 5 times in sample buffer and samples containing 12 μg of protein were added to each well. The solid arrows (→) indicate the position of inner membrane proteins which copurify with intermediate density and outer membrane material following 42°C treatment of cells. The open arrow (⇨) indicates the position of a new protein band which appears in these fractions from 42°C-treated cells. The molecular weight standards mixture(S) contained phosphorylase A (94,000), bovine serum albumin (68,000), gamma globulin heavy chain (50,000), ovalbumin (43,000) and gamma globulin light chain (23,500).


I
II
S
A
B
C
D
A
B
C
D
94K
68K
50K
43K
23.5K

caused adjacent regions of inner membrane to become associated solely with the outer membrane. This occurred only upon heating intact cells as there was no change in the pattern of protein bands observed by SDS-PAGE from any of the membrane fractions when they were heated at 42°C immediately prior to separation on sucrose gradients (Appendix 9).

A new protein band with an apparent molecular weight of 41,000 (Fig. 16, open arrow) appeared in the intermediate density and outer membrane preparations of 42°C-treated cells. This band did not represent a heat-sensitive outer membrane protein as heating membrane fractions at 42°C prior to isolation on sucrose gradients or at 42°C or 100°C prior to application to the gel did not have a similar effect. This band appeared only as a result of heating intact cells and therefore may represent a heat labile protein of inner membrane origin.

Discussion

Following treatment at 42°C, formerly-competent *A. vinelandii* are fully capable of binding donor DNA in a DNase-resistant state but have lost the ability to transport DNA across the cell envelope. Coincident with this loss of transport ability is the heat-sensitive loss of membrane plateaus revealed by freeze-etch techniques. These plateaus represent adhesion sites (Bayer and Leive, 1977) and their absence in noncompetent cells, appearance with competence development, and reappearance upon competence recovery in 42°C-treated cells suggests that they may be competence specific. These sites are not formed simply as a result of the iron limitation used to induce competence because they are not present in iron-limited noncompetent cells.

The disruption of membrane adhesions required for DNA uptake could account for the heat-sensitive nature of *A. vinelandii* transformation. Alternatively, a protein or protein complex required for DNA uptake may have maintained the integrity of the adhesion site during freeze fracture prior to heating. Consistent with the disruption of adhesion sites is the copurification of three inner membrane proteins with outer membrane fragments following heating. This implies that these proteins were located adjacent to adhesion sites which are involved in DNA uptake. These competence-related adhesion sites represent only a small fraction of the cell's adhesion sites, the majority of which appear stable to 42°C treatment.

A pore structure possessing a hydrophilic core may exist at the adhesion site to allow DNA transport. Such a pore has been proposed by Reusch and Sadoff (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K190, p. 168) which is formed of poly-β-hydroxybutyrate and is of sufficient diameter (13Å) to allow uptake of single stranded DNA. The results of these studies cannot be taken as support for this theory as poly-β-hydroxybutyrate was present in the membrane fractions, at least primarily, as contaminating storage granules. The evidence that *A. vinelandii* take up DNA in a double stranded form (Chapter IV) casts further doubt on the function of such a pore.

The mechanism of DNA uptake during genetic transformation is poorly understood. Most models proposed for this process (Akrigg et al., 1969; Erickson, 1970; Grinius, 1980; Lacks, 1977) are not applicable to Gram-negative organisms, including *A. vinelandii* and *H. influenzae*, which take up DNA in a double stranded form in the absence of high concentrations of divalent cations (Biswas and Sparling, 1981; Stuy, 1965). A model for DNA uptake by competent *Bacillus subtilis* proposes DNA binding and internalization by mesosomes which are partially extruded at the growing regions of the cell envelope (Akrigg et al., 1969). Although this hypothesis has been supported by later studies (Vermeulen and Venema, 1974a,b), the mechanism of DNA transport across the membrane bilayer has not been determined. A somewhat analogous situation may exist in competent *H. influenzae* and *H. parainfluenzae* in which specific DNA binding sites are located on membrane blebs which extend from the cell surface (Concino and Goodgal, 1982; Deich and Hoyer, 1982; Kahn et al., 1982). In *H. parainfluenzae* these membrane blebs, containing DNA bound in a DNase-resistant state, appear to be internalized resulting in DNA uptake (Kahn et al., 1982). Although these membrane blebs were not observed to be internalized by *H. influenzae*, they were observed to be adjacent to points of adhesion between the inner and outer membranes which may have some role in DNA uptake obviating a need for internalization of membrane vesicles.

The hypothesis that adhesion sites could form the basis for the molecular architecture of a DNA transport system is an attractive one. It is consistent with the stable association of specific receptors at adhesion sites and the ability of adhesion sites to permit the uptake of double stranded (bacteriophage) DNA (Bayer, 1979; Bayer and Starkey, 1972; Tomita et al., 1976). DNA transport at adhesion sites simplifies DNA uptake to transport across a single hydrophobic region rather than requiring two transport systems for successive uptake across two membrane bilayers. Also, adhesion sites appear to pass through gaps, of reported diameter 10 nm (Leive and Davis, 1980), in the peptidoglycan (Muhlradt and Golecki, 1975). Interestingly, such a hole in the peptidoglycan is similar to the 8 nm diameter "pore" observed in competent *H. parainfluenzae* beneath the DNA binding membrane blebs (Kahn et al., 1982). It is interesting to speculate that calcium, which is known to be necessary for natural membrane fusion (Poste and Allison, 1973) and to promote the fusion of phospholipid vesicles (Verkeij et al., 1979), may also induce membrane adhesion in Gram-negative bacteria which are made competent by treatment with high concentrations of calcium (Cohen et al., 1972).

Acknowledgements

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VI. A Regularly Arrayed Surface Layer of *Azotobacter vinelandii*

Summary

Washing competent *Azotobacter vinelandii* OP strain UW1 with Burk buffer, or heating cells at 42°C, exposed a regularly arrayed surface layer which was effectively revealed by freeze-etch electron microscopy. The layer was composed of tetragonally arranged subunits separated by a center to center spacing of approximately 9 to 10 nm. Cells treated to expose the regular surface layer retained the major envelope protein, a 60,000 molecular weight (60K) glycoprotein. Extraction of most of the 60K glycoprotein, and small amounts of twelve other proteins, by distilled water washing resulted in a disappearance of the regular surface array. Six of these minor proteins were extracted by washing cells with 0.2 M $MgSO_4$; a treatment which left the regular surface layer intact, but as a linear array, and left the 60K glycoprotein cell-bound. The regular surface array was not apparent on competent cells grown in calcium-limited medium although these cells possessed large amounts of the 60K glycoprotein. A surface array appeared on these cells following the addition of calcium to 0.5 mM, concurrent with a large increase in competence. These results suggested that the 60K glycoprotein, in association with calcium ions, was primarily, if not solely, responsible for the formation of the regular surface array. An uninterrupted surface array was not absolutely required for competence nor was it required for normal levels of DNase-resistant DNA binding.

Introduction

The major envelope protein of *Azotobacter vinelandii* is a 60,000 molecular weight (60K), acidic (pI 5.1) glycoprotein which accounts for 20% of total envelope protein of vegetative cells (Page and Doran, 1981; Schenk and Earhart, 1981; Schenk et al., 1977). It copurifies with outer membrane fragments prepared by French pressure cell disruption of cells (Page and von Tigerstrom, 1982) but not with outer membrane fragments released from spheroplasts unless the suspending buffer contains calcium, magnesium, manganese, barium, or zinc at a concentration of 2 mM (Schenk and Earhart, 1981). The 60K glycoprotein is one of the more prominent proteins found in culture supernatants and is released in greater amounts into the growth medium of cells grown under calcium-limited conditions (Page and Doran, 1981). These results suggest that the 60K glycoprotein is associated with the cell surface via salt bridging. This hypothesis is supported by the ease with which it is extracted into distilled water (Page and Doran, 1981; Schenk and Earhart, 1981; Schenk et al., 1977). The 60K glycoprotein shows a strong affinity for calcium and strontium as evidenced in its precipitation from aqueous solution made 20 mM with either divalent cation. Magnesium did not substitute in this regard nor would it substitute for calcium or strontium in the glycoprotein-mediated competence recovery in calcium-limited cells (Page and Doran, 1981). The results of this investigation suggest that the 60K glycoprotein, in association with divalent cations, forms a regularly-arranged surface layer. This study reexamines the role of the 60K glycoprotein in transformation competence in light of these findings.

(Preliminary results of this study were reported at the 83rd Annual Meeting of the American Society for Microbiology, 6 to 11 March 1983, New Orleans, LA.)

Experimental Procedures

Bacterial strains and growth conditions

The transformation recipient *A. vinelandii* OP strain UW1 (Nif$^-$ capsule$^-$ (Fisher and Brill, 1968)) and the DNA donor strains *A. vinelandii* ATCC 12837 strain 113 (Nif$^+$ capsule$^+$) and *A. vinelandii* OP strain UW (Nif$^+$ capsule$^-$) were maintained on Burk medium (Page and Sadoff, 1976) as previously described (Page and von Tigerstrom, 1979). Liquid cultures were grown with aeration by shaking at 170 rpm in a water bath gyrotory shaker model G-76 (New Brunswick Scientific Co., New Brunswick, N.J.). Burk buffer, pH 7.2, was Burk medium without added glucose and ammonium acetate.

Transformation

Competent cells of strain UW1 prepared by 20 to 24 h growth in iron-limited Burk medium (Page and von Tigerstrom, 1978) were transformed as described (Page and von Tigerstrom, 1979) with saturating concentrations of crude lysate DNA prepared from strain 113 (Page and Sadoff, 1976). Transformation frequency was the number of Nif$^+$ transformants detected per number of viable cells selected on Burk medium.

Freeze-etch electron microscopy

Cells for freeze-etch electron microscopy were prepared from fresh cultures and were freeze-etched the same day. All cells were washed once by centrifugation (10,000 rpm) with 1 volume of 5 mM potassium phosphate buffer, pH 7.2, prior to freezing in liquid Freon and freeze-etching as described in Chapter II. The replicas were examined in a Phillips 300 electron microscope operating at an accelerating voltage of 60 or 80 kV.

Washing competent cells

Cells from a competent culture of strain UW1 were washed once by centrifugation (10,000 rpm) with 0.1 times the original volume of Burk buffer, pH 7.2, to remove pigments and other contaminating materials originating from the culture supernatant. The pellets were then washed 5 times (washes 1 to 5) with 0.2 volume of Burk buffer, distilled water, or 0.2 M $MgSO_4$-50 mM tris (hydroxymethyl) aminomethane (Tris), pH 7.8, and vigorous vortex mixing. The cell pellets were saved for freeze-etch electron microscopy. Similar wash fluids were combined and sterilized by filtration (0.45-μm Millipore filter). The wash fluids were dialyzed twice in 48 h against 20 volumes of distilled water at 4°C. The 0.2 M $MgSO_4$-50 mM TrisHCl wash fluids were made 100 mM with EDTA prior to dialysis. The samples were lyophilized to dryness and resuspended in distilled water prior to analysis of protein content by isoelectric focusing. Protein samples were similarly prepared from distilled washes of noncompetent cells of strain UW1.

Isoelectric focusing

Isoelectric focusing (IEF) in polyacrylamide vertical slab gels was carried out according to Page and Doran (1981). IEF tube gels (2 mm diameter) were prepared from the same gel mixture as slab gels with additional 2% Triton X-100 (Chapter II). Protein bands were stained as described by Fairbanks et al. (1971) with the modification that Coomassie blue R250 concentrations were decreased by 60% to reduce background staining. The pH gradient across the gels was determined as described previously (Page and Doran, 1981).

[^{32}P]DNA binding

Cells from a portion of a competent culture were washed as described above ("washing competent cells") and resuspended in Burk buffer containing 8 mM $MgSO_4$ (transformation assay buffer [Page and von Tigerstrom, 1979]). Washed and unwashed cells were exposed to a two to threefold excess of [^{32}P]DNA prepared from strain UW as previously described (Chapter II). After 10 min incubation at 30°C, the cells were pelleted, washed once in one volume of transformation assay buffer and resuspended in one volume of this buffer. Following treatment of cells with DNase 1 (final concentration 4 μg/ml) for 10 min at 30°C, the cells were washed as before and resuspended in saline citrate buffer, pH 7.2, containing 0.05% sodium dodecyl sulfate. The cells were lysed by heating for 60 min at 60°C. The radioactivity of the samples was determined by liquid scintillation counting as described previously (Chapter IV).

Competent cells prepared by growth in calcium- and iron-limited medium were exposed to 0.5 mM $CaSO_4$ for 30 min or were washed five times with 0.2 volume of distilled water to remove the 60K glycoprotein (Page and Doran, 1981). The ability of treated and untreated cells to bind [^{32}P]DNA in a DNase-resistant state was then determined.

Chemicals

Chemicals for electrophoresis were obtained from Bio-Rad Laboratories (Mississauga, Ontario) except for acrylamide and N,N'-methylene bisacrylamide which were from Eastman Kodak Co. (Rochester, N.Y.) and ampholytes which were supplied by LKB (Fisher Scientific Co., Edmonton, Alberta). All other chemicals were reagent grade and most were purchased from Sigma Chemical Co. (St. Louis, Mo.).

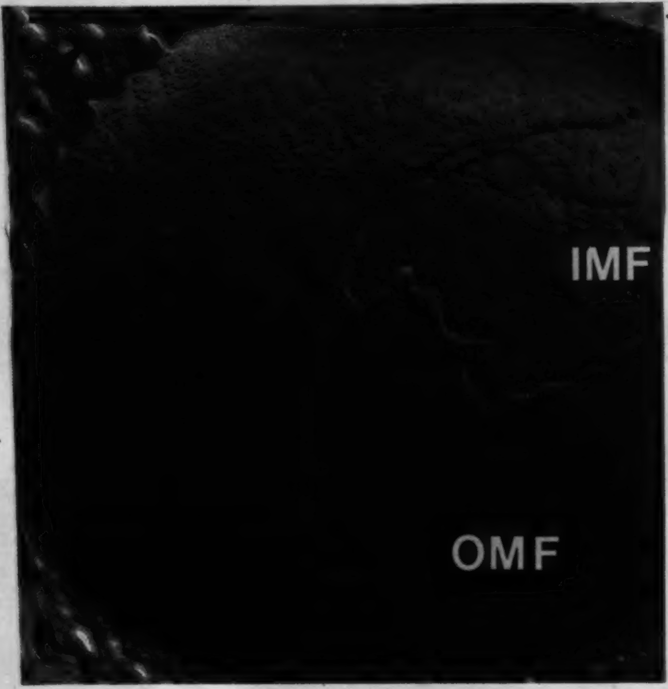
Results

Exposure of regular surface layer

Examination of untreated, competent cells by freeze-etch electron microscopy failed to reveal any distinctive features on the external cell surface (Fig. 17). Pretreating a competent culture at 42°C for 20 to 30 min prior to the preparation of cells for freeze-etching exposed a regularly arrayed layer on the outer convex surface (Fig. 18). Examination of replicas of cells which had exposed convex fracture planes both within the hydrophobic core, and on the outer surface, of the inner and outer membranes confirmed the location of the regularly

Figure 17. Freeze-etch replica of the convex surface of a cell from a competent population of strain UW1 (Nif$^+$ transformation frequency 1.6×10^{-2}). The abbreviations are those recommended by Bayer and Lieve (1977): outer membrane fracture face, OMF; inner membrane fracture face, IMF. The direction of platinum shadowing is indicated by $\theta\rightarrow$. Bar represents 0.5 μm.

Figure 18. Freeze-etch replica of the convex surface of a cell from a 42°C-treated, formerly-competent culture. RS indicates the regular surface layer. The arrows indicate regions of discontinuity in the regular surface layer. Other symbols and abbreviations were defined in Fig. 17. Bar represents 0.5 μm.


IMF
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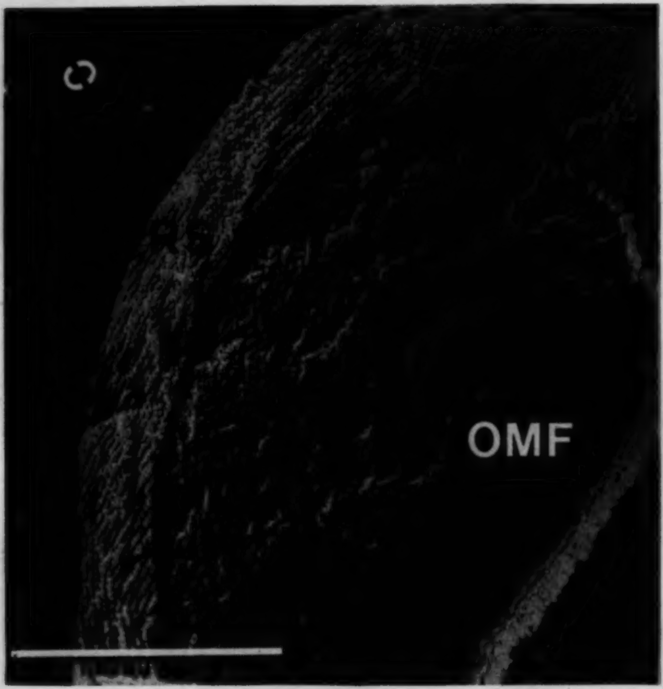

OMF

arrayed layer external to the outer membrane (Chapter V, Fig. 11b). The regular surface layer was also exposed by washing cells with Burk buffer at 30°C (Fig. 19) although it was more effectively revealed when the buffer temperature was increased to 42°C (Fig. 20).

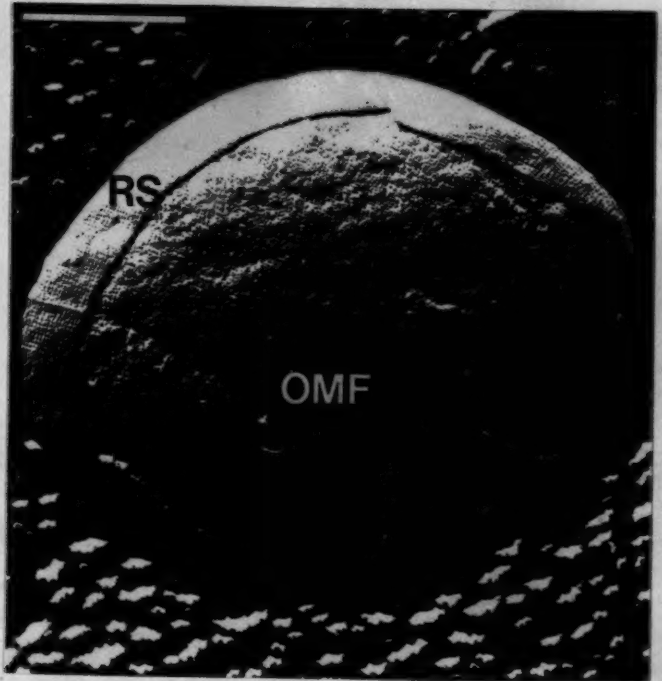
The regular surface layer was formed of tetragonally arranged subunits organized in rows at an angle approximately 90° to each other. The center to center spacing between tetragonal groupings of subunits was estimated to be 9 to 10 nm. Although no gaps in the layer were detected in the freeze-etch replicas, the regular surface array did not appear as a single continuous sheet. It was often interrupted by one or more discontinuities present where a region of the layer organized in one orientation became contiguous with a region of lattice oriented at a different angle (Fig. 18).

Protein content of wash fluids

It had been previously proposed that the 60K glycoprotein might form a regular surface layer (Page and Doran, 1981; Schenk and Earhart, 1981; Schenk et al., 1977). Analysis of the protein content of Burk buffer wash fluids by isoelectric focusing in polyacrylamide gels demonstrated that washing cells at 30°C failed to remove detectable amounts of any envelope protein whereas treatment at 42°C removed three proteins exhibiting isoelectric points of 5.7, 5.8 and 6.3 (Fig. 21). Comparison of these bands to a band containing the 60K glycoprotein (pI 5.1 (Appendix 1)) demonstrated that the 60K glycoprotein was not removed by Burk buffer washing. It had been shown previously that there was minimal release of the 60K glycoprotein during treatment of cells

Figure 19. Freeze-etch replica of the convex surface of a Burk buffer washed (30°C) cell. Abbreviations and symbols were defined in Figs. 17 and 18. Bar, 0.5 µm.

Figure 20. Freeze-etch replica of the convex surface of a Burk buffer washed (42°C) cell. Abbreviations and symbols were defined previously. Bar, 0.5 µm.


RS
OMF

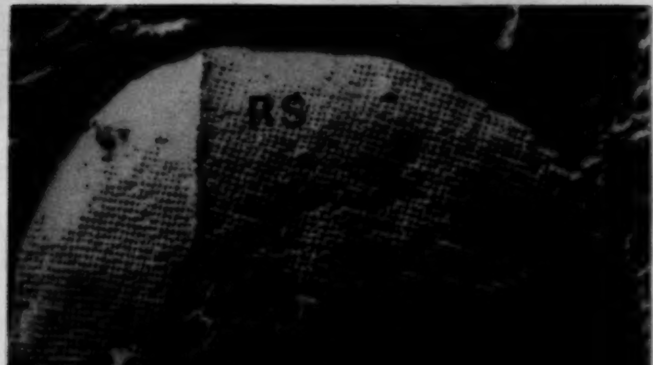
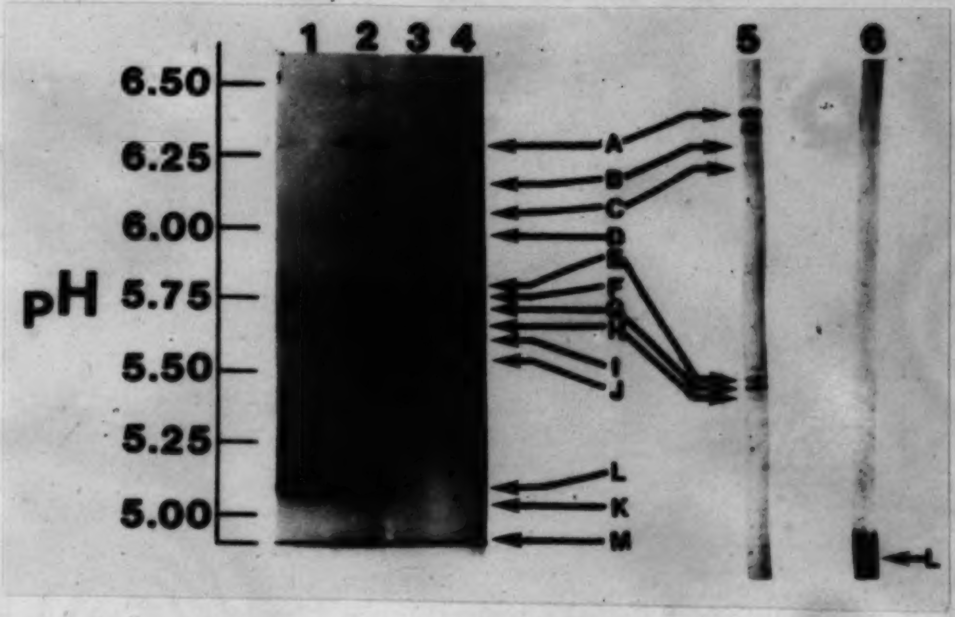

RS
OMF

Figure 21. IEF-PAGE of proteins present in wash fluids. Proteins obtained by washing cells from equivalent portions of a competent culture of strain UW1 (Nif$^+$ transformation frequency 6.9 x 10^{-3}) with Burk buffer or distilled water at 30°C or 42°C (see also Fig. 25) were concentrated as described (Experimental Procedures) and redissolved in equivalent volumes of distilled water. Samples of 50 μl each prepared from 42°C distilled water wash fluids (Lane 2), 30°C Burk buffer wash fluids (Lane 3) and 42°C Burk buffer wash fluids (Lane 4) were applied to the slab gel. A preparation of 60K glycoprotein concentrated from 30°C distilled water washes of Burk buffer grown strain UW1 (Page and Doran, 1981) was applied to lane 1 of the IEF slab gel (80 μg protein) and to the IEF tube gel designated lane 6. Proteins concentrated from 0.2 M $MgSO_4$-50 mM TrisHCl, pH 7.8, washes of competent strain UW1 were prepared separately and the amount of protein applied to the IEF tube gel (designated lane 5) bears no relationship to the amount of protein added to lanes 2-4. The bands detected in lane 2 by Coomassie blue staining were designated A to M.


1
2
3
4
5
6
6.50
6.25
6.00
pH
5.75
5.50
5.25
5.00
A
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D
E
F
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H
I
J
L
K
M
L

at 42°C (Chapter IV, Fig. 7). Therefore the treatments utilized to expose the regular surface layer had not removed significant amounts of 60K glycoprotein.

The removal of the 60K glycoprotein by distilled water washing is well documented (Page and Doran, 1981; Schenk and Earhart, 1981; Schenk et al., 1977). IEF-PAGE analysis of the protein content of distilled water wash fluids obtained by washing competent cells at 42°C confirmed the presence of a single prominent band (pI 5.1) corresponding to the 60K glycoprotein (Fig. 21). This analysis also demonstrated the removal of 12 minor proteins; an observation not previously reported. The presence of residual 60K glycoprotein on the surface of 42°C distilled water washed cells was detected by [I^{125}]-labeling of intact cells and analysis of total cell protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Page and von Tigerstrom, 1982) and gel autoradiography (W. Bingle, unpublished data). Examination of these cells by freeze-etch electron microscopy failed to reveal the regular surface array (Fig. 22) despite the fact that the cells had been heated at 42°C and that distilled water washing was expected to be at least as effective as buffer washing at removing any obscuring material. It was surmised, therefore, that the regular surface layer had been removed concomitant with extraction of the 60K glycoprotein. The external cell surface represented in Fig. 22 was assumed, therefore, to be the outer surface of the outer membrane.

Competent strain UW1 were also washed with 0.2 M $MgSO_4$-50 mM TrisHCl; a treatment similar to that used to release alkaline phosphatase from *Pseudomonas aeruginosa* (Cheng et al., 1970). Examination of these cells by freeze-etch electron microscopy revealed that the regular surface layer was present but the characteristic tetragonal organization was replaced by a strictly linear array (Fig. 23). Examination of several replicas confirmed that this appearance was not due to the particular angle of platinum shadowing. Electrophoretic analysis of the protein content of the wash fluids verified that the 60K glycoprotein had not been removed (Fig. 21). Nine proteins were extracted including six which were determined, by comparison of isoelectric points, to be equivalent to proteins removed by distilled water washing at 42°C.

Effect of wash treatments on competence

The effect of certain wash treatments on transformation competence is illustrated in Fig. 24. These treatments did not reduce cell viability. Repeatedly washing cells with Burk buffer had no specific detrimental effect on competence. The initial decrease in competence occurred whenever cells were centrifuged and resuspended (Appendix 3). The effects of washing cells at 42°C cannot be readily distinguished from the rapid loss of competence incurred by heating at 42°C (Chapter IV, Fig. 5). The deleterious effects of washing cells with distilled water at 30°C varied from the four or five fold reduction in competence pictured in Fig. 24 to a total loss of competence. Regardless of the final level of competence, this treatment removed a considerable amount of the 60K glycoprotein (Fig. 25). Examination of these cells by freeze-etch electron microscopy failed to reveal a regular surface array.

Figure 22. Freeze-etch replica of the convex surface of a distilled water washed (42°C) cell. Abbreviations and symbols were defined in Fig. 17 except OMO which indicates that portion of the replica corresponding to the outer surface of the outer membrane. Bar, 0.5 μm.

Figure 23. Freeze-etch replica of the convex surface of a cell which had been washed at 30°C with 0.2 M $MgSO_4$ in 50 mM TrisHCl, pH 7.8. Abbreviations and symbols were defined in Figs. 17 and 18. Bar, 0.5 μm.

OMO

OMF

Figure 24. Effect of wash treatments on competence. A competent culture of strain UW1 (Nif+ transformation frequency 1.5 x 10^{-3}) was divided into four equal portions. Each portion was washed once in 0.1 volume of Burk buffer prior to washing five times (washes 1 to 5) in 0.2 volume of 30°C Burk buffer (○), 42°C Burk buffer (□), 30°C distilled water (●), or 42°C distilled water (△). Competence was assayed following each of the final five washes.

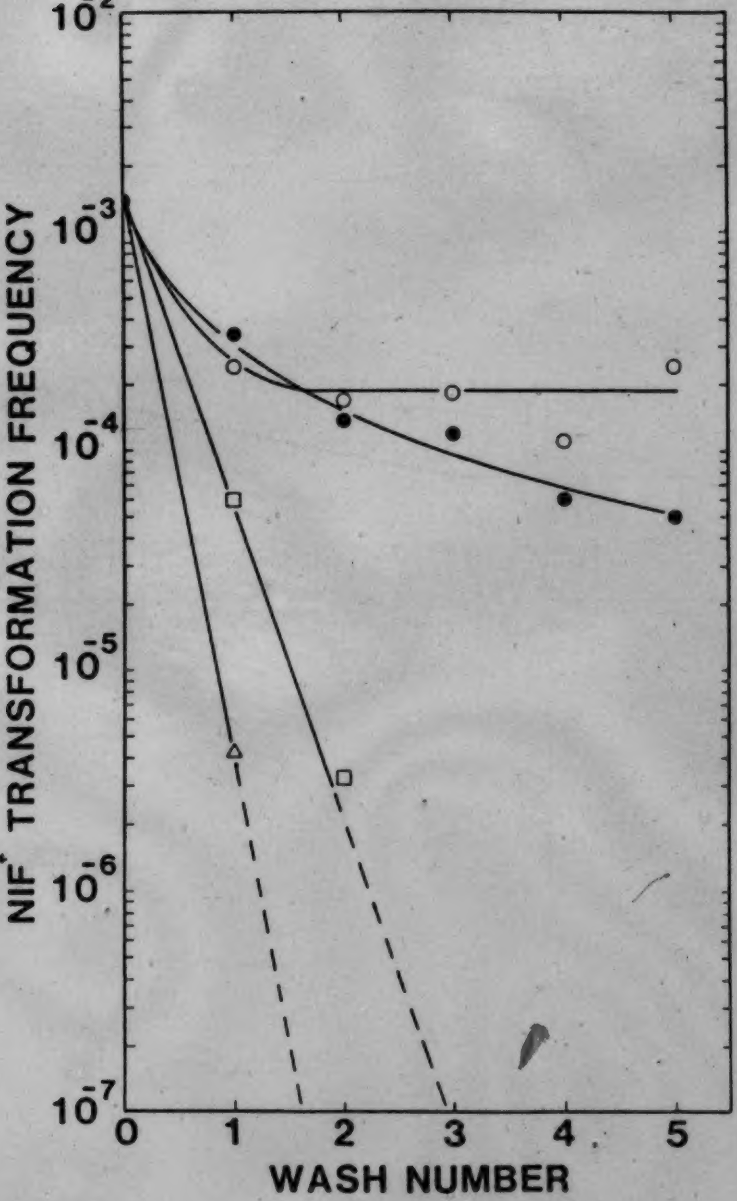
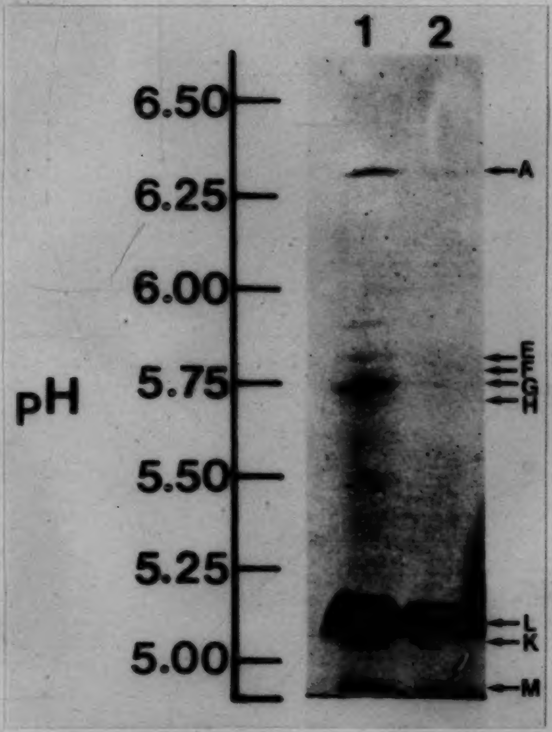

10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
NIF^+ TRANSFORMATION FREQUENCY
0
1
2
3
4
5
WASH NUMBER

Figure 25. IEF-PAGE of proteins released into 30°C distilled water wash fluids. Proteins obtained by washing cells from a portion of a competent culture (see Fig. 21) with 0.2 volume of distilled water at 30°C were concentrated (Experimental Procedures) and dissolved in a volume of distilled water equivalent to that used for the samples listed in Fig. 21. A 50 μl sample was applied to lane 1 of the slab gel. Lane 2 contained a preparation 60K glycoprotein (85 μg protein) concentrated from 42°C distilled water washes of Burk buffer grown strain UW1. The letters used to designate each protein band indicate the correspondence of these bands to the protein bands presented in Fig. 21.


1
2
pH
6.50
6.25
6.00
5.75
5.50
5.25
5.00
A
E
F
G
H
L
K
M

Washing competent strain UW1 five times with 0.2 volume of 0.2 M $MgSO_4$-50 mM TrisHCl destroyed competence with little or no reduction in the numbers of viable cells. Competence recovered in this population following incubation in original culture supernatant (Fig. 26). This redevelopment of competence, like competence development in untreated cells (Fig. 26), required protein synthesis.

Effect of calcium on the regular surface array

The 60K glycoprotein was involved in calcium-mediated competence recovery in calcium- and iron-limited strain UW1 (Page and Doran, 1981). Cells isolated from a calcium- and iron-limited population (Nif^+ transformation frequency 3.0×10^{-4}) were examined by freeze-etch electron microscopy before and after (Nif^+ transformation frequency 4.7×10^{-3}) the culture was made 0.5 mM with $CaSO_4$. The regularly-ordered array was not observed on the outer surface of cells prior to the addition of $CaSO_4$ (Fig. 27) but was present afterward (Fig. 28). The regular surface array was observed, though only faintly, on calcium-treated cells without prior treatment to remove any obscuring material indicating that at least some of this material was lost concurrent with the loss of a variety of envelope proteins (Page and Doran, 1981). This suggested that treating calcium-limited cells at 42°C was not necessary in order to reveal a regular surface layer. Confirmation of this awaits further experimentation. Although calcium-limited cells released greater amounts of 60K glycoprotein into the growth medium than calcium-sufficient cells (Page and Doran, 1981), analysis of outer membranes from both cell types by sodium dodecyl

Figure 26. Competence recovery in cells washed with 0.2 M $MgSO_4$. Cells pelleted from aliquots of a competent culture of strain UW1 (Nif^+ transformation frequency 5.3×10^{-3}) were washed five times with 0.2 volume of 0.2 M $MgSO_4$-50 mM TrisHCl, pH 7.8, after which no competent cells were detected. The cells were then resuspended in the original volume of filter-sterilized (0.45 µm Millipore filter) culture supernatant prepared as described (Chapter IV) from the original competent culture. Equal volumes of the treated culture (open symbols) were added to two Erlenmeyer flasks. Equivalent volumes of the original competent culture (closed symbols) were similarly added to two other Erlenmeyer flasks. Chloramphenicol (final concentration 50 µg/ml) was added to one of the treated (△) and untreated (▲) cultures. After 30 min incubation at 30°C, the cells were pelleted from the four cultures, washed once in one volume of filter-sterilized original, competent, culture supernatant and resuspended in one volume of this supernatant. Incubation was continued and at time intervals aliquots were removed for transformation assay.

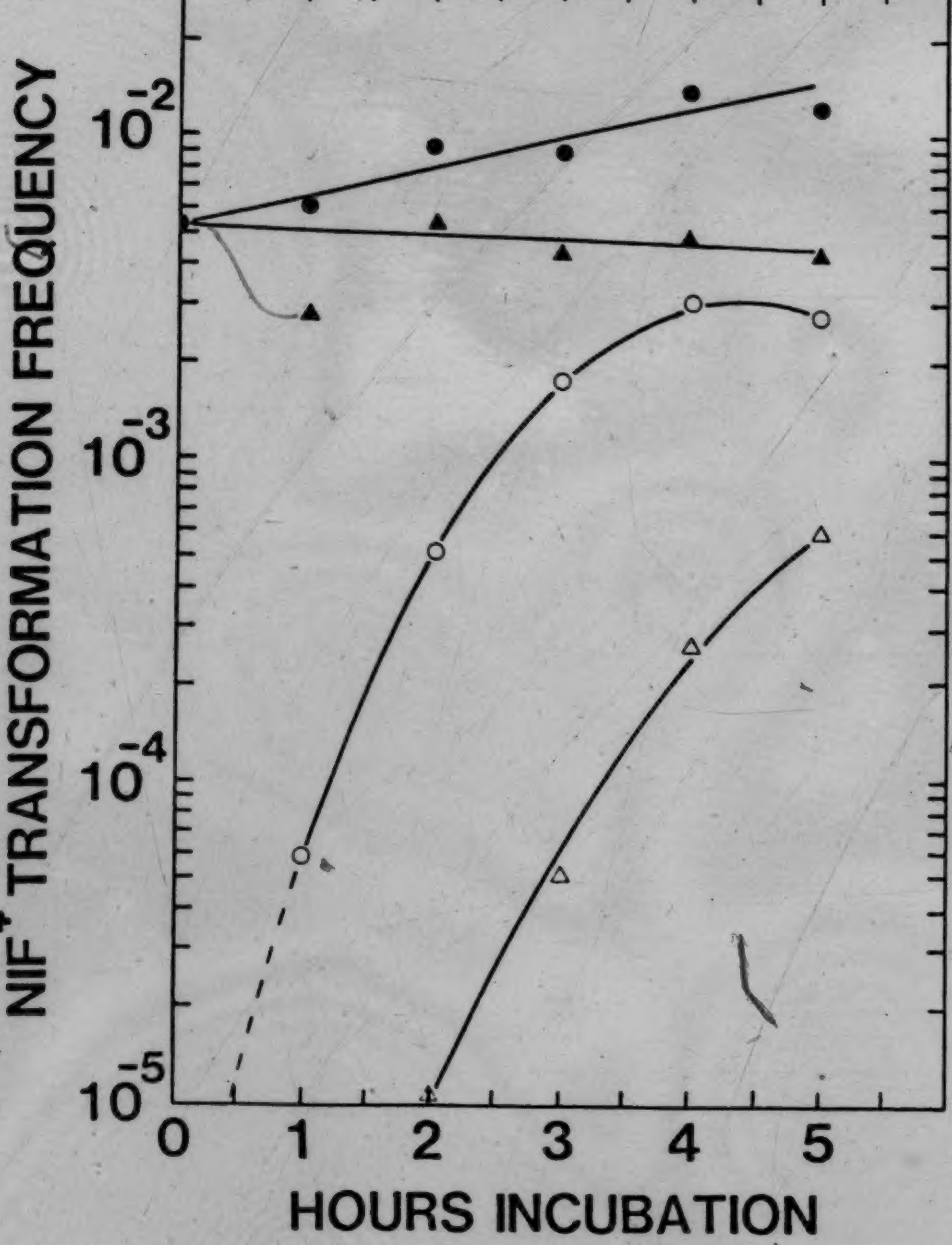
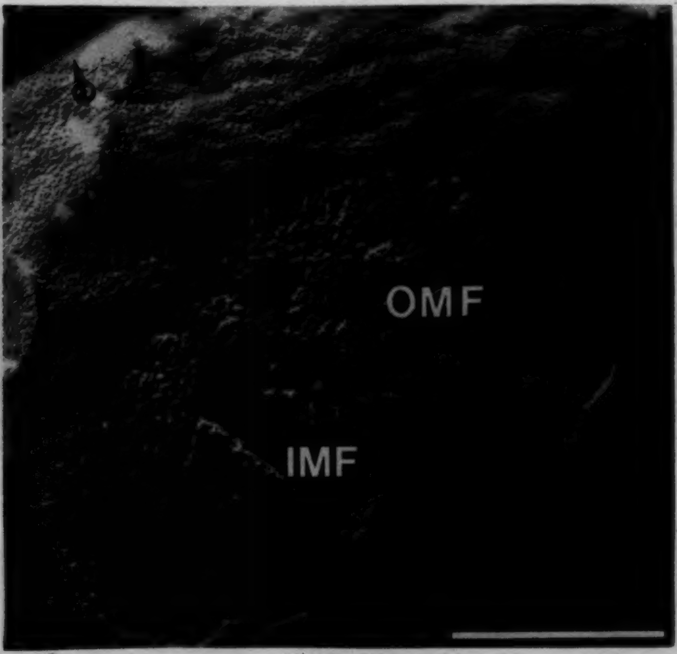

NIF$^+$ TRANSFORMATION FREQUENCY
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
1
2
3
4
5
HOURS INCUBATION

Figure 27. Freeze-etch replica of the convex surface of a cell grown in calcium- and iron-limited Burk medium. The symbols and abbreviations were defined previously. Bar, 0.5 μm.

Figure 28. Freeze-etch replica of the convex surface of a cell exposed to 0.5 mM $CaSO_4$ for 30 min following growth in calcium- and iron-limited Burk medium. The symbols and abbreviations were previously defined. Bar, 0.5 μm.


OMF
IMF

BS

sulfate-polyacrylamide gel electrophoresis failed to indicate depletion of 60K glycoprotein from the envelopes of calcium-limited cells (W. Bingle, unpublished data). Therefore, it appeared that calcium promoted the organization of bound 60K glycoprotein into a regular lattice rather than reattachment of the 60K glycoprotein onto the cell surface.

DNase-resistant DNA binding

The ability of competent cells to bind [^{32}P]DNA in a DNase-resistant state was compared with that of competent cells treated to expose, disorder or remove the regular surface array (Table 7). Included in this comparison were poorly competent cells prepared by growth in calcium- and iron-limited medium. These cells were washed with distilled water to remove the 60K glycoprotein (Page and Doran, 1981) or exposed to 0.5 mM $CaSO_4$ in order to organize the 60K glycoprotein into a regular array. The results presented in Table 7 indicate that the amount of [^{32}P]DNA bound to the cells in a DNase-resistant state bore no relationship to the level of competence in the population or the presence or absence of the regular surface layer or the 60K glycoprotein.

Discussion

Regularly arranged surface layers are a component of the cell envelope of a number of Gram-negative, Gram-positive and Gram-variable bacteria (for review see Sletyr, 1978). The majority of these extrinsic arrays are formed from a single species of protein or glycoprotein

TABLE 7. DNase-Resistant [^{32}P]DNA Binding to Competent and Noncompetent Strain UW1.

Recipient	Treatment[a]	Nif$^+$ Transformation[a] Frequency	60K Glycoprotein[b]	Tetragonal Surface[b] Array	cpm / 10^8 cells[a,c]
Fe-limited cells	none	2.7×10^{-2}	present	present	5.1×10^4
	30°C BB[d] wash	2.2×10^{-2}	present	present	5.0×10^4
	42°C BB wash	ND[d]	present	present	1.1×10^4
	0.2 M $MgSO_4$-50 mM Tris wash	ND	present	disordered (linear array)	6.8×10^4
	30°C dH_2O[d] wash	8.3×10^{-2}	very little present	absent	5.9×10^4
	42°C dH_2O wash	ND	very little present	absent	5.4×10^4
Fe- and Ca-limited cells	none	3.5×10^{-4}	present	absent[e]	5.8×10^4
	exposure to 0.5 mM $CaSO_4$	1.1×10^{-3}	present	present	4.2×10^4
	30°C dH_2O wash	ND	absent or little present[f]	not determined	7.2×10^4

[a] The procedure for the treatment of competent cells and the methods of determining DNase-resistant [^{32}P]DNA binding and transformation frequency were described in Experimental Procedures.

[b] The presence or absence of the 60K glycoprotein, or the regularly arrayed layer, on the cell surface was determined by IEF-PAGE analysis of wash fluids and freeze-etch electron microscopy, respectively.

[c] The results of a typical experiment using a single set of samples.

[d] Abbreviations: BB, Burk buffer, pH 7.2; dH_2O, distilled water; ND, not determined.

[e] Based on preliminary data (see text).

[f] From Page and Doran, 1981.

with a molecular weight ranging from 48,000 to 150,000. These molecules are typically self-assembled into a monolayer of hexagonally, tetragonally or linearly arranged subunits with a center to center spacing varying from 4 to 25 nm.

The tetragonal array of particles, separated by a center to center spacing of about 9 to 10 nm, on the surface of *A. vinelandii* OP strain UW1 represents a typical regular surface layer. Similar to the tetragonally-arranged surface layers of *Bacillus sphaericus* (Howard and Tipper, 1973) and *Clostridium thermosaccharolyticum* (Sletyr and Thorne, 1976), the regular surface layer of strain UW1 was composed of several tetragonal arrays which met along discontinuous borders. These faults in the regular array may represent the "migrating dislocations" proposed by Harris and Scriven (1970) to be the sites of insertion of newly synthesized molecules.

It was fortuitous that the regular surface array of strain UW1 was exposed simply by pelleting cells following treatment at 42°C. It is doubtful that the amorphous appearance of the outer surface of untreated cells was due to a layer of eutectic material (DeVoe et al., 1971) since the Burk buffer washed, and untreated, competent cells were similarly washed and resuspended in phosphate buffer prior to freezing. Furthermore, 42°C treatment was a more effective means of exposing the regular surface layer than Burk buffer washing at 30°C which may have been more likely to remove loosely associated, contaminating materials from the cell surface. It is possible that the small amount of capsular material produced by strain UW1 (W. Page, in press, Can. J. Microbiol.) was obscuring the regular surface layer of untreated cells.

The correlation between the presence of the 60K glycoprotein on the cell surface and the appearance of the regular surface array, established using various wash treatments, suggests that the 60K glycoprotein is the regular surface layer component. Consistent with this hypothesis is the relatively large abundance of this glycoprotein in the cell envelope (Schenk et al., 1977) and the apparently uniform distribution of the 60K glycoprotein over the cell surface (Schenk and Earhart, 1981). Whether or not the 60K glycoprotein is the sole component forming a homogenous layer cannot be determined from these data. One or more of the twelve minor proteins removed from cells, concurrent with extraction of the 60K glycoprotein into distilled water, may be a minor component of the tetragonal array. Since six of these twelve proteins, including the most prominent ones, were removed by treatment of cells with 0.2 M $MgSO_4$ (which reorganized the layer into a linear array), it is conceivable that one or more of these six proteins promoted the formation of a tetragonal array. It is equally reasonable to suppose that the removal of these proteins was indicative of the disorganization or disruption of the outer membrane effected by high magnesium ion concentration or by treatment with Tris or both (Ingram et al., 1971). The partial disorganization of the regular surface layer, therefore, may have occurred in response to disruption of the underlying layer (Buckmire and Murray, 1970); which is presumably the outer membrane.

The 60K glycoprotein appears to be associated with the underlying layer via salt linkages mediated by divalent cations (Page and Doran, 1981; Schenk and Earhart, 1981). Displacement of the 60K glycoprotein by EDTA or distilled water washing (Page and Doran, 1981; Schenk and Earhart, 1977; Schenk et al., 1981) verifies such ionic linkage. The fact that several divalent cations promote this association (Schenk and Earhart, 1981) suggests a certain lack of specificity in this interaction similar to that observed with the divalent cation associated surface layers of *Spirillum serpens* (Buckmire and Murray, 1973) and *Acinetobacter* (Thorne et al., 1975).

The interaction between 60K glycoprotein molecules appears to be specifically mediated by calcium or strontium (Page and Doran, 1981). Preliminary results suggest that $CaSO_4$ promotes the ordering of the 60K glycoprotein, bound to the surface of calcium-limited cells into a regular array. Since the growth medium contained 0.8 mM $MgSO_4$ (Page and Sadoff, 1976), it was a lack of calcium rather than a lack of sulfate which resulted in a disordered array. Furthermore, it appears as though magnesium is unable to substitute for calcium in ordering the array although magnesium probably mediated glycoprotein binding to the underlying layer. This sort of specific calcium-mediated protein-protein interaction to form a lattice (Buckmire and Murray, 1973, 1976; Beveridge and Murray, 1976) and reversible reassembly of a regular array (Beveridge and Murray, 1976; Thorne et al., 1975) have been previously reported for other regular surface layers. If it is true that the interactions between glycoprotein molecules in the regular array of strain UW1 are mediated by calcium ions but not magnesium ions, then the conversion of the tetragonal array to a linear array following the treatment of cells with 0.2 M $MgSO_4$ may be the result of calcium displacement by magnesium. The fact that the regular array was not completely disordered suggests that only certain calcium ions were able to be displaced. This phenomenon of disordering of a regular surface array through cation substitution has been observed with other organisms (Beveridge, 1979; Beveridge and Murray, 1976).

The 60K glycoprotein bears striking similarities to the tetragonal surface array component of *Acinetobacter* MJT/F5/199A (Thornley et al., 1974). Both molecules are equally abundant in the cell envelope, have a similar molecular weight and contain a similar proportion of acidic and basic amino acid residues. They are both extractable with EDTA and both appear to associate with the cell surface via salt bridging mediated by any one of several divalent cations. As well, both appear to be ordered into a regular array by a specific divalent cation which also may be used to precipitate the proteins from aqueous solution. Furthermore, both components are abundant in the growth medium of cultures despite the fact that the cells appear to possess uninterrupted surface arrays. It is suggested that cells overproduce the regular surface layer component specifically to ensure the existence of an uninterrupted layer (Sletyr and Glauert, 1976).

The biological importance of the regular surface layer formed by the 60K glycoprotein has not been determined, however, it has been suggested that regular surface layers may have an important role in conditioning the environment to which the outer membrane is directly exposed (Sletyr, 1978; Beveridge, 1981).

The role of the 60K glycoprotein in competence for genetic transformation remains uncertain. Although an uninterrupted superficial array represents a potential barrier to the penetration of DNA, these surface layers are known to be tolerant to interruptions introduced by such structures as flagella (Sletyr and Glauert, 1975) and therefore specific DNA binding receptors may protrude through the layer. Alternatively, the lack of trypsin-sensitivity of competence (Chapter IV) may be accounted for by specific DNA receptors which underlie the regular surface layer. This would imply that the layer is porous to transforming DNA. The layer is necessarily porous to small nutrient molecules (Schenk and Earhart, 1981) and, being a highly polar array, would be expected to contain aqueous channels. If these channels between subunits were as large as the 2 nm diameter pores reported in the regular surface layer of *Sporosarcinae ureae* (Stewart and Beveridge, 1980), they would permit the passage of DNA.

The regular surface layer was not necessary to provide a surface charge compatible for DNA binding as previously suggested (Page and Doran, 1981) nor was it essential for protection of bound DNA from digestion by externally added DNase. This does not mean that it may not serve these functions in vivo but rather it may indicate that more than one layer of the cell envelope may adequately provide for DNase-resistant DNA binding, as is the case with *Escherichia coli* (Weston et al., 1981). Competent *A. vinelandii* participate in two types of DNase-resistant DNA binding (Chapter IV). The predominant form involves a nondiscriminating DNA binding to the cell surface which does not necessarily lead to transformation. A second form, specific for homologous DNA, appears to be an early step in DNA uptake for genetic transformation. Since cells which lacked a majority of the 60K glycoprotein were poorly competent, it is possible that these cells participated primarily in the former type of DNase-resistant DNA binding.

A role for the 60K protein in transformation competence has been demonstrated (Page and Doran, 1981). Further support for this theory is derived from the observation that 42°C distilled water washed cells retained some 60K glycoprotein and therefore increases in competence which previously were attributed solely to the addition of calcium ions (Page and Doran, 1981) may have involved an interaction between calcium ions and the 60K glycoprotein. Calcium-mediated recovery of competence in calcium-limited cells appeared to involve an ordering of bound 60K glycoprotein into a regular array rather than reassembly of this glycoprotein onto the cell surface. Whether or not a restructuring of the surface array promotes a necessary organization of the underlying layer or better allows DNA access to DNA binding receptors involved in DNA uptake cannot be discerned. The effects of the wash treatments or growth conditions used to remove or disorder the regular array do little to delineate the role of the 60K glycoprotein in competence. Growth in calcium-limited medium not only results in a disordered surface array but also causes the release of a large variety of envelope proteins (Page and Doran, 1981), any one of which may be involved in competence. Similarly, washing cells at 30°C with distilled water removes the 60K glycoprotein and reduces competence but also may have radically perturbed the cell membranes by extraction of phospholipid (Smith and Wyss, 1969) or lipopolysaccharide (W. Bingle, unpublished data).

The total loss of competence incurred by washing cells with 0.2 M $MgSO_4$ in Tris buffer has not been previously reported. Although a number of membrane proteins were removed by this treatment and protein synthesis was required for competence, these results cannot be interpreted to indicate that one or more of these proteins were required for competence as this treatment may have extracted lipopolysaccharide and generally perturbed the organization of the outer membrane (Ingram et al., 1973).

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VII. Concluding Discussion

Bacteria which are naturally induced to competence for genetic transformation have developed a unique capacity for genetic adaptation. *Azotobacter vinelandii* was only recently demonstrated to possess this ability (Page and Sadoff, 1976a).

The physiological events occuring during competence development, and during DNA binding and uptake by competent bacteria, are poorly understood (Lacks, 1977a; Smith et al., 1981; Venema, 1979). However, competence development in the best-characterized bacterial transformation systems is known to involve changes occurring in the cell envelope which allow for the binding and transport of DNA (e.g. Deich and Hoyer, 1982; Joenje et al., 1975; Kahn et al., 1982; Lacks and Neuberger, 1975; Mulder and Venema, 1982a,b; Seto et al., 1975). These relatively preliminary studies of the *Azotobacter* transformation system were determined, therefore, to be primarily concerned with the early events in the transformation process.

In order for *Azotobacter vinelandii* to be transformed, exogenous DNA must permeate the capsule, the regular surface layer, the outer membrane, the peptidoglycan layer and the cytoplasmic membrane. Strains of *A. vinelandii* which produce large amounts of capsular material are poorly transformable (Page and Sadoff, 1976a), presumably due to an inability of most DNA to diffuse through the capsule. *A. vinelandii* OP strain UW1 produces very little capsular material (Fisher and Brill, 1969; W. Page, in press, Can. J. Microbiol.) and serves as an excellent transformation recipient.

A. vinelandii strain UW1 was demonstrated to possess a regular surface layer external to the outer membrane similar to the closely related bacteria, *Azomonas agilis* (Houwink, 1963) and *Azomonas insignis* (Glauert, 1962). This tetragonally arranged layer is easily distinguishable from the apparently hexagonally arranged layer determined from certain freeze-etch replicas of *A. vinelandii* to exhibit a center to center spacing between subunits of only 6 to 8 nm (Cagle et al., 1972). This latter array is similar in appearance to the regular lattice formed on the outer membrane of *Escherichia coli* by the so-called matrix protein (Beveridge, 1981; Rosenbusch, 1974). Interestingly, *A. vinelandii* may possess an outer membrane protein equivalent to the *E. coli* matrix protein (Page and von Tigerstrom, 1982). The inability of Cagle et al. (1972) to correctly identify the various fracture planes observed in their freeze-etch replicas allows one to speculate that the hexagonal array component of *A. vinelandii* may be a type of matrix protein. The tetragonal array examined in this study is composed primarily from repeating molecules of an acidic, 60K glycoprotein.

There is some evidence, discussed earlier, which suggests that the linkage of the 60K glycoprotein to the underlying layer may be mediated by any of several divalent cations whereas the salt-bridging between 60K glycoprotein molecules required to organize a lattice may specifically require calcium ions. Proof of this latter hypothesis may require that purified glycoprotein molecules, in the presence of calcium ions alone, be shown capable of forming a lattice, in vitro, which is visibly indistinguishable from the regular array on the cell surface. A very similar experiment using the surface layer component from *Acinetobacter* has proven successful (Thornely et al., 1974). The evidence for a role of the regular surface layer in genetic transformation has come primarily from studies of the calcium requirement for competence induction.

Competent cells of strain UW1 prepared in iron-limited Burk medium lacking calcium were shown to be poorly transformable. The growth rate of these cells was comparable, however, to that of cells grown in competence induction medium containing 0.6 mM calcium. Therefore, the effects of calcium-limitation, unlike magnesium-limitation (Arancia et al., 1980; Fiil and Branton, 1969; Stan-Lotter et al., 1979), did not include a drastic impairment of cell metabolism. The effects of calcium-limitation appeared, for the most part, to have been limited to a loss of integrity in various fractions of the cell envelope.

The exposure of calcium- and iron-limited cells to 0.5 mM calcium promoted an increase in the level of competence of the culture varying from one to three orders of magnitude. Since either calcium chloride or calcium sulfate promoted competence recovery, it is likely that the calcium ions, not the counter ions, were primarily responsible for the increase in competence. Calcium-mediated competence recovery in calcium-limited cells which had been washed twice with distilled water and resuspended in buffer was much less than that of unwashed cells resuspended either in buffer or in original culture supernatant. Therefore, calcium-mediated competence recovery did not involve the synthesis of some component necessary for transformation. Although calcium-limited cells released a variety of proteins into the growth medium, either during growth or during the centrifugation of cells from the medium, none of these proteins were necessary for calcium-mediated competence recovery. Competence recovered equally well in unwashed cells whether they were resuspended in the original culture supernatant or in iron-limited Burk buffer. Therefore, calcium ions must interact with some cell bound component which, directly or indirectly, influences the transformation process. Several lines of evidence support the hypothesis that this component is the 60K glycoprotein.

Although the presence of culture supernatant was not necessary for calcium-mediated competence recovery in unwashed cells, some factor or factors in the growth medium did promote competence recovery in distilled water washed cells. This factor is presumably common to the distilled water wash fluids and to the original culture supernatant. It also must be present in noncompetent culture supernatants since they were capable of supporting competence recovery in distilled water washed, calcium-limited cells. The 60K glycoprotein proved to be the common factor.

The affinity of the 60K glycoprotein for calcium ions is obvious from the demonstration that these glycoprotein molecules rapidly precipitate from aqueous solution made 20 mM in calcium ions. Although such an acidic glycoprotein might be expected to interact with divalent cations, the specificity of this interaction is evidenced by the observation that only one other divalent cation tested, namely strontium, was able to substitute for calcium in this regard. It is probably significant that the two types of divalent cations which were capable of ionically bridging between 60K glycoprotein molecules in solution also promoted competence recovery, whereas magnesium ions were not active in either process.

There is precedence for surface layer components, which associate with the cell surface via divalent cation mediated ionic linkage, to assemble onto an appropriate cell surface deficient in this component provided the appropriate divalent cation is present (Beveridge and Murray, 1976b; Thorne et al., 1975). Purified 60K glycoprotein, in the presence of calcium ions, promoted at least a partial recovery of competence in distilled water washed cells, presumably by such a process of reassembly. Complete competence recovery may have been prevented by the perturbation, or extraction into distilled water, of other envelope components necessary for transformation. Alternatively, the aggregation of 60K glycoprotein molecules in solution may have competed with the reassembly of the glycoprotein onto the cell surface thereby preventing the formation of sufficiently large tracts of regular surface layer to promote a large increase in competence.

Preliminary evidence suggests that calcium-limited cells fail to possess a regular surface array although there is an abundance of cell-bound 60K glycoprotein. The regular surface array was certainly present on cells following the calcium-mediated recovery of competence. This implies that it was not the presence of cell-bound glycoprotein, per se, which promoted competence recovery but rather it was the calcium-mediated formation of a regular surface array.

Whether the regular surface layer is intimately involved in the early stages of the transformation process or whether it is a relatively inert layer compatible with the diffusion of DNA to the outer membrane remains to be determined. As mentioned previously, the supposition that specific DNA receptors, which presumably include envelope proteins, underlie the regular surface layer may account for the lack of competence loss in trypsin-treated cells. If this were true then the regular surface layer must be porous to molecules of transforming DNA. One can extrapolate from this hypothesis and suggest that calcium-limited cells may be poorly transformable because the 60K glycoprotein molecules are not sufficiently organized to allow the formation of the relatively large aqueous channels necessary for the DNA to gain access to the outer membrane. Furthermore, the acidic 60K glycoprotein molecules present as a highly ordered array may be less inclined to interact with the negatively charged DNA molecules via the magnesium ions present in the transformation assay and thereby hinder the involvement of this DNA in genetic transformation.

Recently, the nature of the regular surface layer component of strain UW1 has come into question. It has become apparent that a protein of approximate molecular weight 55,000 to 60,000 may be the surface layer subunit whereas this protein and a glycoprotein of an apparent molecular weight of 60,000 are both present in culture supernatants (W. Bingle, unpublished data; W.H. Bingle, J.L. Doran, and W.J. Page, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K155, p. 202). This would explain the appearance of two major protein bands migrating close together on polyacrylamide gels when culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2) or isoelectric focusing (Fig. 3). Typically, only one major band was detected when distilled water wash fluids containing the regular surface layer component were similarly analyzed (Figs. 2, 21, 25). Since no functional significance was attributed to the carbohydrate portion of the 60K glycoprotein, this new finding has relatively little bearing on the previous interpretation of the results.

In the presence of magnesium ions and excess DNA, competent cells bound DNA in both a DNase-resistant and DNase-sensitive state. The treatment of these cells with DNase did not diminish the eventual transformation frequency indicating that it was the DNA bound in a state insensitive to externally added DNase which was destined to transform the cells. The discovery that treating competent cells at 42°C would dissociate the process of DNA uptake from the earlier stages of the transformation process allowed a separate examination of DNase-resistant DNA binding. It is of interest to note that the discovery of methods for experimentally dissecting the transformation process are especially important in studies of *A. vinelandii* since it is very difficult to generate mutants of *Azotobacter* in genes other than those involved in nitrogen-fixation (Mishra and Wyss, 1968; Sadoff et al., 1979; Terzaghi, 1980) and thereby genetically dissect the transformation process.

DNase-resistant DNA binding to strain UW1 is distinct from DNA uptake into the cytoplasm. Competent cells appeared to participate in two forms of DNase-resistant DNA binding. One form of binding, proposed to involve "type 1" DNA receptors, demonstrated the characteristics of a relatively nonspecific association of DNA with the cell surface. This form of DNase-resistant DNA binding was not saturated at concentrations of DNA in excess of that required to saturate the cells in terms of transformation. Although this type of DNA binding occurred in the absence of transformation, DNA bound in this manner may have sterically-hindered other DNA species from becoming active in transformation. The only apparent requirement for this form of DNA binding was the presence of magnesium ions.

The nature of the magnesium requirement for DNase-resistant DNA binding is uncertain. If DNA molecules are required to permeate a highly polar regular surface layer, then magnesium ions might facilitate transformation by properly neutralizing the negative charge on the DNA. DNase-resistant DNA binding did not require the presence of a regular surface array although during the process of extracting this layer the cells became poorly transformable. It can be concluded, therefore, that at least DNA binding to some form of type 1 receptor was not prevented by the loss of this layer. The majority of the DNA which was bound to cells in a DNase-resistant state but not transported into the cytoplasm was removed from cells concurrent with the formation of spheroplasts. It may be likely, therefore, that most of the DNA which was not involved in the transformation process remains associated with the outer membrane. This suggests that DNA binding to type 1 receptors may simply represent an association of DNA to the negatively charged outer surface of the outer membrane (Costerton et al., 1974) sufficient to convey DNase-resistance. Magnesium ions could mediate such an association. This sort of nonspecific association of DNA with the outer membrane may account for the lack of discrimination by type I receptors against the DNase-resistant binding of heterologous DNA. It also may account for the ability of competent, precompetent and noncompetent cells of strain UW1 to bind large amounts of DNA in a DNase-insensitive state; a characteristic apparently unique to *A. vinelandii*.

As discussed previously, type 2 receptors are defined as those DNA binding sites which were responsible for the saturable DNase-resistant DNA binding which occurred as a specific step preliminary to DNA uptake. The simple saturation kinetics of the DNase-resistant DNA binding leading to transformation suggests that type 2 receptors may be discrete entities. A single type 2 receptor may be available to bind only one molecule of DNA since cells previously exposed to saturating concentrations of DNA, in the absence of the ability to transport this DNA, were unable to participate in further DNase-resistant DNA binding leading to transformation.

Competent cells of strain UW1 discriminate against transformation by heterologous DNA. The ability of heterologous DNA species to block transformation by homologous DNA roughly correlated with the ability of these DNA species to serve as a transforming principle. Unlike transforming DNA species, the ability of nontransforming DNA to block transformation by homologous DNA did not vary with the time of exposure of cells to blocking DNA prior to the addition of homologous DNA. It is assumed from this observation that nontransforming DNA fails to become involved in the transformation process proper. If DNA binding to type 2 receptors is the initial stage of what can be specifically considered to be the transformation process, then these receptors may be responsible for discriminating against the uptake of heterologous

DNA. It may be unlikely that the lack of transforming activity of heterologous DNA can be attributed to the failure of this DNA to possess the appropriate modification pattern since attempts to demonstrate restriction enzyme activity in extracts of *A. vinelandii* were unsuccessful (W. Page and K. Roy, unpublished data). If the mechanism of discrimination involved is analogous to that of other Gram-negative organisms which are naturally induced to competence, then it may entail that type 2 receptors are capable of recognition of a specific DNA sequence (Danner et al., 1980; Graves et al., 1982; Mathis and Scocca, 1982; Smith and Sisco, 1979).

Two non-bacterial DNA species, salmon sperm DNA and ØW-14 DNA, were tested for their ability to prevent the transformation of strain UW1 by homologous DNA (Appendix 6). Surprising observations were obtained in both cases. Salmon sperm DNA was ineffective as a blocking DNA even when present in amounts fivefold greater than the amount of homologous DNA. Therefore salmon sperm DNA did not affect transformation by homologous DNA even on the basis of a dilution of the homologous DNA marker being selected for in transformed cells. This implies that those nontransforming DNA species which were capable of blocking transformation by homologous DNA did not do so by a dilution effect.

ØW-14 DNA proved to be a more effective competing DNA than any other heterologous DNA tested. Similar results were obtained when ØW-14 DNA was used to inhibit the genetic transformation of *Bacillus subtilis* (Lopez et al., 1982; Lopez et al., 1980a). The samples of ØW-14 DNA differed from other samples of competing DNA in two respects. The samples, themselves, contained EDTA and the DNA molecules were unique in that approximately fifty per cent of the thymine residues had been replaced by putrescinylthymine (Kropinski et al., 1973). The maximum final concentration of EDTA in the transformation assays involving ØW-14 DNA was 0.05 mM. This concentration of EDTA was found to be insufficient to have any effect on transformation by homologous DNA (data not shown). Whether or not the putresine residues bestowed upon the DNA an increased affinity for some element of the DNA binding, uptake or integration mechanisms of strain UW1 is not known.

Clearly, it is advantageous for competent strain UW1 to have a mechanism which allows cells to take up homologous DNA preferentially. As pointed out by Smith et al. (1981), this encourages the uptake of only that DNA which is likely to be integrated into the host chromosome and therefore be of value to the cell. Moreover, it is not unexpected that there is some risk involved in taking up whatever DNA molecules the cells come in contact with in their environment especially considering that this may result in transfection rather than transformation. In this regard, a mechanism for the recognition of a particular DNA sequence rather than a DNA base-modification pattern is particularly important as only the former mechanism would discourage transfection by bacteriophage DNA which had replicated in cells of the same strain. There remains, however, a finite possibility that the particular recognition sequence, or a mildly degenerate form of it, may exist on heterologous DNA molecules (Smith et al., 1981). This may account, for instance, for the low level of transformation of *A. vinelandii* by *Rhizobium* DNA. Perhaps for this reason, *A. vinelandii* and *H. influenzae* are not constitutively amenable to genetic transformation but only become so under the harsh conditions imposed by nutrient deprivation (Herriott et al., 1970; Page, 1982; Page and von Tigerstrom, 1978).

The fact that cells of strain UW1 discriminate against transformation by heterologous DNA, including certain plasmid cloning vectors maintained in *E. coli*, prevents the use of these highly competent cells as hosts for common cloning vehicles. Strain UW1 could prove to be very useful as a host for recombinant DNA (for reasons stated in the Introduction (Chapter I)) but this will apparently require that plasmids native to *Azotobacter* be developed for use as cloning vectors. To date, there is no conclusive evidence for the existence of such plasmids despite the fact that an interest in determining whether the genes for nitrogen fixation in *Azotobacter* might be borne on a plasmid, similar to those of some *Rhizobium* species (Masterton et al., 1982; Nuti et al., 1979; Prakash et al., 1981), has caused several investigators to search for one (P.E. Bishop; W.J. Brill; personal communications). Although two plasmids, of apparent molecular weights 2.7×10^7 and 2.6×10^8, have been reportedly isolated from *A. vinelandii* ATCC 12837 (R.L. Slot, R.N. Reusch, and H.L. Sadoff, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H22, p. 116) attempts to demonstrate the existence of a plasmid in strain UW1 using several procedures for the isolation of large plasmids (Casse et al., 1979; Crosa and Falkow, 1981; Hirsch et al., 1980; Kado and Liu, 1981) were unsuccessful. However, when DNA isolated from a population of transformed cells of strain UW1 was analyzed by agarose gel electrophoresis, a band of DNA was observed migrating somewhat slower than the main band chromosomal DNA (Appendix 5). Efforts are underway to determine whether this band represents plasmid DNA.

Currently, heterologous plasmid DNA can only be used to transform *A. vinelandii* made competent by the artificial induction method used to develop competent *E. coli* (Cohen et al., 1972; David et al., 1981). Clearly, the process of transformation in these cells is different from that in cells of *A. vinelandii* which are naturally induced to competence. Not only do the two systems differ in their compatibility to transformation by heterologous DNA but they also differ in their response to high concentrations of calcium and to temperature. The artificial induction of competence requires that cells are treated with 200 mM calcium chloride (David et al., 1981) whereas one-tenth this concentration of calcium chloride in the competence induction medium prevented the natural development of competent cells. The development of the ability for artificially induced competent cells of *A. vinelandii* to transport DNA required that the cells be pretreated at 42°C (Bergmans et al., 1981; Weston et al., 1981). This same treatment had exactly the opposite effect on cells of strain UW1 which were naturally grown to competence.

During the transformation of *A. vinelandii* which are naturally induced to competence, approximately fifteen to twenty per cent of the DNA bound to competent cells in a DNase-resistant state was transported into the cell cytoplasm. The transforming DNA did not appear to enter a phase of biological eclipse during the transformation process since biologically active donor DNA marker was recovered in the envelope-free crude lysate of cells approximately one minute after these cells were exposed to transforming DNA. Although the amount of donor DNA marker recoverable from the cytoplasm of competent cells increased over the 90 min period immediately following the initial mixing of cells with DNA, the amount of recoverable marker decreased during the subsequent 90 min period (data not shown). This suggests that shortly after uptake the donor DNA marker had not attained a permanent status in the cell. Therefore, the presence of biological activity probably was not the result of a rapid integration of the DNA following uptake. The observation that heat-denatured DNA would not serve as a transforming principle, therefore, suggests that cells of strain UW1 take up DNA in a double-stranded form similar to other Gram-negative bacteria which are naturally induced to competence (Barhart and Herriott, 1963; Biswas and Sparling, 1981; Notani and Goodgal, 1966; Stuy, 1965). This notion is supported by the observation that, during the uptake of radioactively-labelled DNA, the cells of strain UW1 did not release an equivalent amount of acid-soluble radioactivity similar to bacteria which are known to convert transforming DNA to a single-stranded form prior to or during uptake (Lacks, 1977a).

The treatment of competent strain UW1 at 37 to 42°C resulted in a loss of the ability of cells to transport transforming DNA across the cell envelope. This did not appear to be due to the inhibition of synthesis of some component required for transport which was rapidly turned over by the cells since there was a 2 h delay before this factor was expressed in cells returned to incubation at 30°C. Rather, there appeared to be some heat-labile structure or function involved in DNA uptake. The results of this study suggest that DNA uptake occurred at certain heat-labile sites of adhesion between the inner membrane and the cell wall.

Certain adhesion sites, which fortuitously were among those revealed by freeze-etch electron microscopy, were observed to be sensitive to heating at 42°C. Obviously, this single observation is insufficient to imply that the loss of these adhesion sites is related to the loss of transformation competence but several other observations do serve to support such an hypothesis. The disruption of these adhesion sites was not simply indicative of a general loss of membrane integrity since most adhesion sites (observed by thin section electron microscopy) were not similarly disrupted and there did not appear to be a general heat-induced loss of membrane material (Katsui et al., 1982). Only a relatively small amount of the regular surface layer component was observed to be released from 42°C-treated cells. The heat-labile adhesion sites were shown to redevelop concurrent with the recovery of competence in 42°C-treated cells. Similarly, these adhesion sites first appeared in cells just prior to the initial development of competence. The development of these adhesion sites was not simply part of the response of cells to iron-limitation (Page and von Tigerstrom, 1978, 1982) since noncompetent cells grown in iron-limited Burk medium containing glutamate as the sole nitrogen source did not possess these structures. Noncompetent cells prepared by growth in Burk medium (containing iron) also failed to possess adhesion sites demonstrable by freeze-etch techniques suggesting that these adhesion sites may be unique to competent cells.

The structures observed in freeze-etch replicas of competent cells which were determined to represent adhesion sites were "plateaus" of outer membrane material situated on a fracture plane which otherwise had passed through the hydrophobic core of the inner membrane (Bayer and Lieve, 1977). It has been suggested that the presence of these structures may be due to certain integral inner membrane proteins which provide sufficient resistance to cleavage through the inner membrane (M.E. Bayer, and M.H. Bayer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, J3, p. 172). However, the observation that certain inner membrane proteins copurified with outer membrane material following the heat-induced disruption of these "plateaus" in strain UW1 implies that these structures do represent points of association between the inner and outer membranes.

Rather surprisingly, perhaps, these apparently competence-specific adhesion sites developed in the absence of synthesis of any competence-specific inner membrane or outer membrane proteins (W. Page and M. von Tigerstrom, 1982; unpublished results). One integral membrane protein of apparent molecular weight 49,000 was specific to the intermediate density membrane fraction of competent cells. This membrane fraction has been suggested to be enriched for adhesion sites (Bayer et al., 1982; Tomita et al., 1976). The relationship between this synthesis of this protein and the development of competence has not been investigated. The development of these membrane adhesion sites did occur concurrent with changes in the fatty acid constituents of the membrane phospholipids (Appendix 10). The major changes in the fatty acid residues of the phospholipids of both the inner and outer membranes during competence development were an increase in the relative amount of palmitic acid and a decrease in the relative amounts of myristic, palmitoleic and oleic fatty acids. Both competent and noncompetent vegetative cells were found to contain small amounts of seventeen and nineteen carbon chain cyclopropane fatty acids. These fatty acids were previously reported to be specific to *A. vinelandii* cysts (Lin and Sadoff, 1969; Sadoff et al., 1975). The very long chain twenty-one and twenty-three carbon chain fatty acids also reported to be specific to cysts of *A . vinelandii* (Su et al., 1981) were not detected in either competent or noncompetent cells. Similar to *H. influenzae* (Sutrina and Scocca, 1976), competence development in *A. vinelandii* strain UW1 occurred in the absence of changes in phospholipid head groups (W. Page and M. von Tigerstrom, unpublished results). Unfortunately, very little is known concerning the structural organization of adhesion sites (Bayer, 1979) and it was not determined which fatty acids were associated with which phospholipid head groups. Therefore it is difficult to correlate changes in the fatty acid content of the phospholipids with the development of the specialized membrane structures possibly existing at the adhesion sites. Once more becomes known about the structure of adhesion sites an investigation of the phospholipid content of the intermediate density membrane fraction from competent and noncompetent cells may yield interesting results.

Competence recovery in 42°C-treated cells required RNA and protein synthesis. If certain adhesion sites are actually the sites of DNA uptake, then it is not unexpected that the insertion of pore-forming or DNA binding proteins at these adhesion sites is necessary for DNA transport. In an attempt to identify such proteins, 42°C-treated cells were labeled with [^{14}C]glucose for a period during competence recovery. Unfortunately, these results failed to indicate the specific synthesis of any integral membrane proteins (Appendix 11). Instead there was a general labeling of most integral membrane proteins in proportion to their prominence in the membrane as determined by Coomassie blue staining.

It could not be determined whether the cells which recovered competence following treatment of a competent population of strain UW1 at 42°C were also competent prior to heating. In populations of other Gram-negative organisms which are naturally induced to competence, virtually all cells are transformable (Biswas et al., 1977; Herriott et al., 1970; Sparling, 1966). Attempts to determine the actual level of competence in a transformable population of strain UW1 by assaying the transformation frequency of single and double transformants by unlinked markers (Goodgal and Herriott, 1961) were unsuccessful. Since *A. vinelandii* are reported to contain 20 to 50 copies of the chromosome per cell (Sadoff et al., 1979), the failure to estimate the level of competence by this method may have been due to the tendency of the two markers present in doubly transformed cells to segregate rapidly.

The timing of events during the genetic transformation of *A. vinelandii* was similar to that in *H. influenzae*. DNase-resistant DNA binding in both organisms demonstrated a lag of 1 to 3 sec and was saturated 5 to 10 min after the exposure of cells to saturating concentrations of DNA (Deich and Smith, 1980; Stuy and Stern, 1964). Although DNase-resistant DNA binding occurred relatively early in the process of genetic transformation of *A. vinelandii*, DNA uptake appeared to extend over at least the 90 min period following initial DNA binding. Newly acquired donor DNA was expressed in cells beginning 60 to 80 min after the exposure of competent cells to DNA. Therefore, the timing of DNA integration in strain UW1 is also comparable to that of *H. influenzae* in which integration occurred between 10 and 60 min after the initial exposure of cells to donor DNA (Stuy, 1965).

In order to understand the early stages of the transformation process in molecular terms it is important to identify and characterize the envelope components involved. Very little progress has been made in this regard.

Similar to *H. influenzae* (Herriott et al., 1970), *A. vinelandii* OP strain UW1 is induced to competence under nongrowth conditions which permitted protein synthesis. Despite this, no competence specific integral inner membrane or outer membrane proteins appear to exist (Page and von Tigerstrom, 1982, unpublished results). This study, however, has indicated that several envelope proteins may be worthy of further investigation in regard to possible roles in genetic transformation.

The extraction into distilled water of one particular acidic envelope protein (pI 5.19) correlated with the inability of calcium-limited cells to recover competence in the presence of calcium ions and culture supernatant. The fact that this protein was not similarly extracted in detectable amounts from noncompetent cells or very poorly competent cells suggests that its presence in strain UW1 may correlate with the existence of a competent state. The difficulty with interpreting these results to imply that this protein may have a role in competence is that the treatment used to extract this protein probably had other detrimental effects on competence resulting from the extraction of phospholipid (Smith and Wyss, 1969) and lipopolysaccaride (W. Bingle, unpublished data). Similarly, it has not been determined whether the release of four common proteins from competent cells washed with distilled water or 0.2 M magnesium sulfate in Tris buffer is related to the loss of competence incurred in each case or whether the release of these proteins is only indicative of a general loss of membrane integrity (Cheng et al., 1970; Ingram et al., 1973; Smith and Wyss, 1969). It is certainly expected that some envelope proteins are involved in DNA binding and uptake. Obviously the formation of specific adhesion sites is insufficient for DNA uptake unless a hydrophilic pore, presumably formed by proteins, also exists at this site. Furthermore, protein molecules have been clearly demonstrated to be capable of the recognition of specific DNA sequences (Anderson et al., 1981; McKay and Steitz, 1981). Therefore, it is possible that a specific envelope protein is responsible for the mechanism of discrimination against the uptake of heterologous DNA by competent cells of strain UW1.

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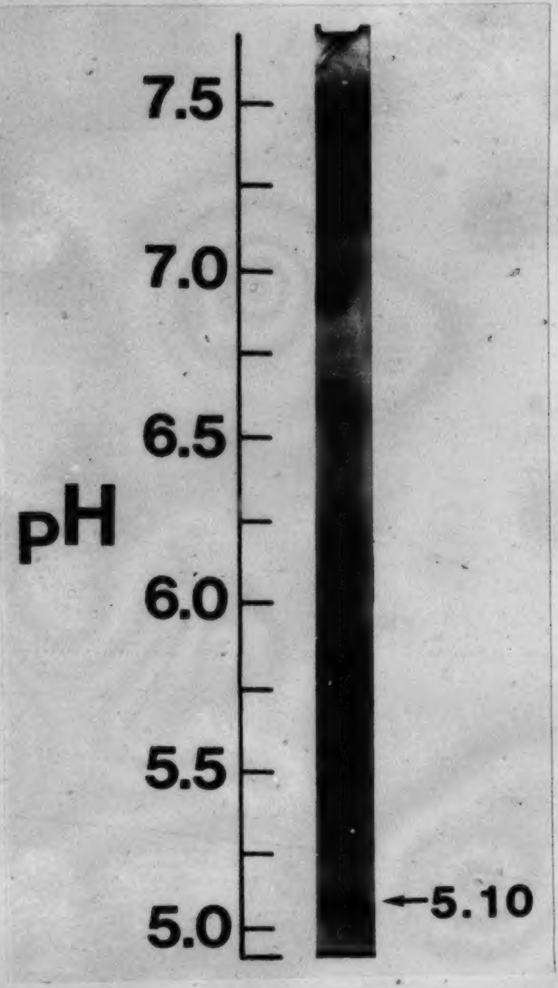
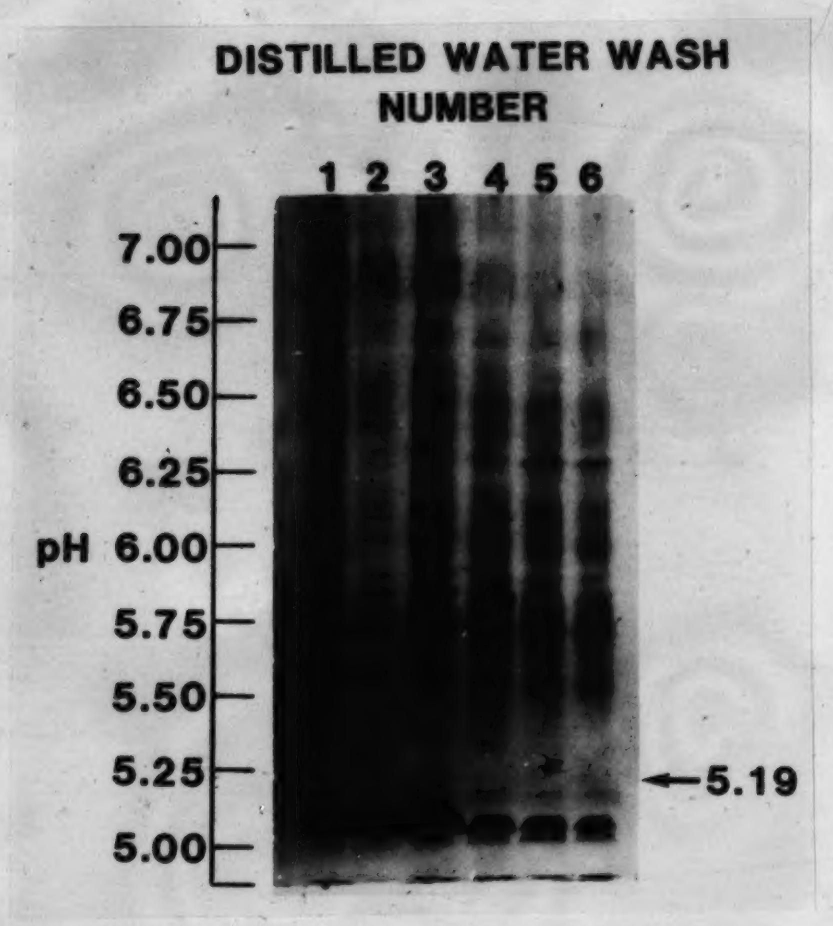
Appendices

Appendix 1. IEF-PAGE of concentrated glycoprotein preparation (Chapter III). 13 μg of protein was added to the gel.


pH
7.5
7.0
6.5
6.0
5.5
5.0
←5.10

Appendix 2. IEF-PAGE of distilled water washes from noncompetent cells prepared in Burk medium lacking calcium. Approximately 3 X 10^{11} cells were washed 6 times with distilled water as described (Page and Doran, 1981). The proteins present in these wash fluids were concentrated according to Page and Doran (1981) and 150 μg of protein was applied to each well of the gel.


DISTILLED WATER WASH
NUMBER
1
2
3
4
5
6
pH
7.00
6.75
6.50
6.25
6.00
5.75
5.50
5.25
5.00
5.19

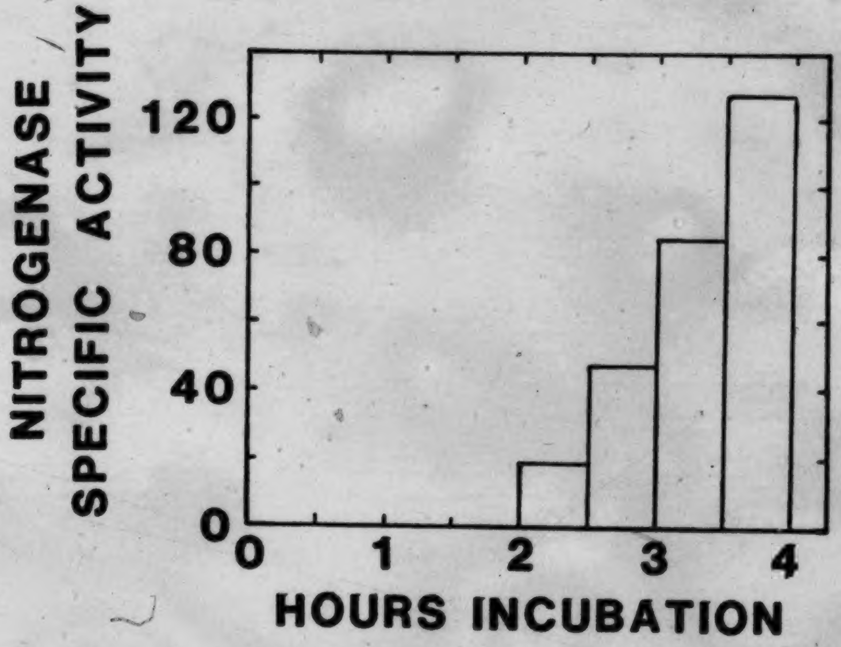
Appendix 3. Competence Recovery by 42°C-Treated Cells in Fresh Medium and Different Growth Supernatants[a]

Recovery Medium	Transformation Frequency
Original supernatant-no resuspension[b]	2.6×10^{-3}
Supernatant from unheated culture	8.6×10^{-4}
Supernatant from 42°C-treated culture	5.3×10^{-4}
Fresh competence induction medium	7.5×10^{-5}

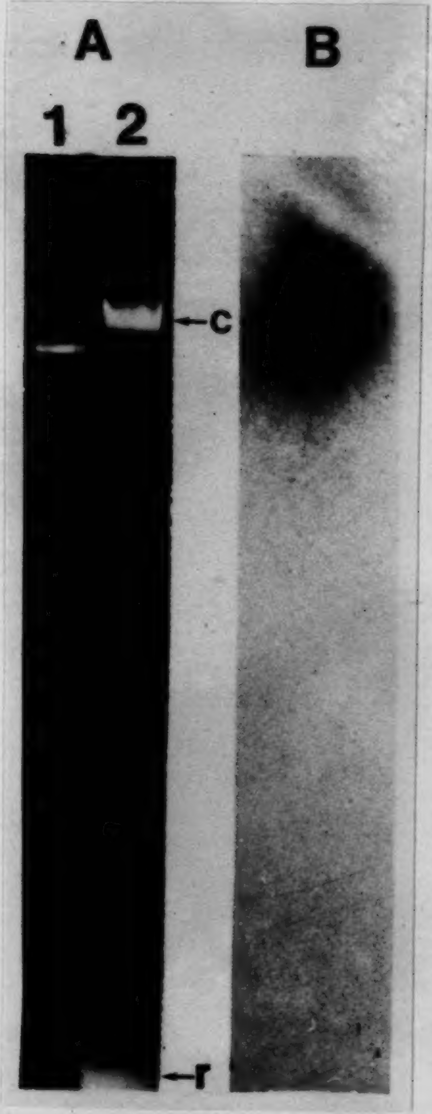
[a] Strain UW1 cells grown to competence (Nif^+ transformation frequency 1.1×10^{-2}) in iron-limited Burk medium (competence induction medium) were treated at 42°C for 25 min after which no competent cells were detected. Cells were harvested from aliquots of this culture by centrifugation and resuspended in an original volume of filter sterilized culture supernatant prepared from the heated and unheated portions of the original culture or in fresh competence induction medium. The level of competence was determined following 5 h incubation at 30°C.

[b] A portion of the 42°C-treated culture was incubated without harvesting and resuspending the cells.

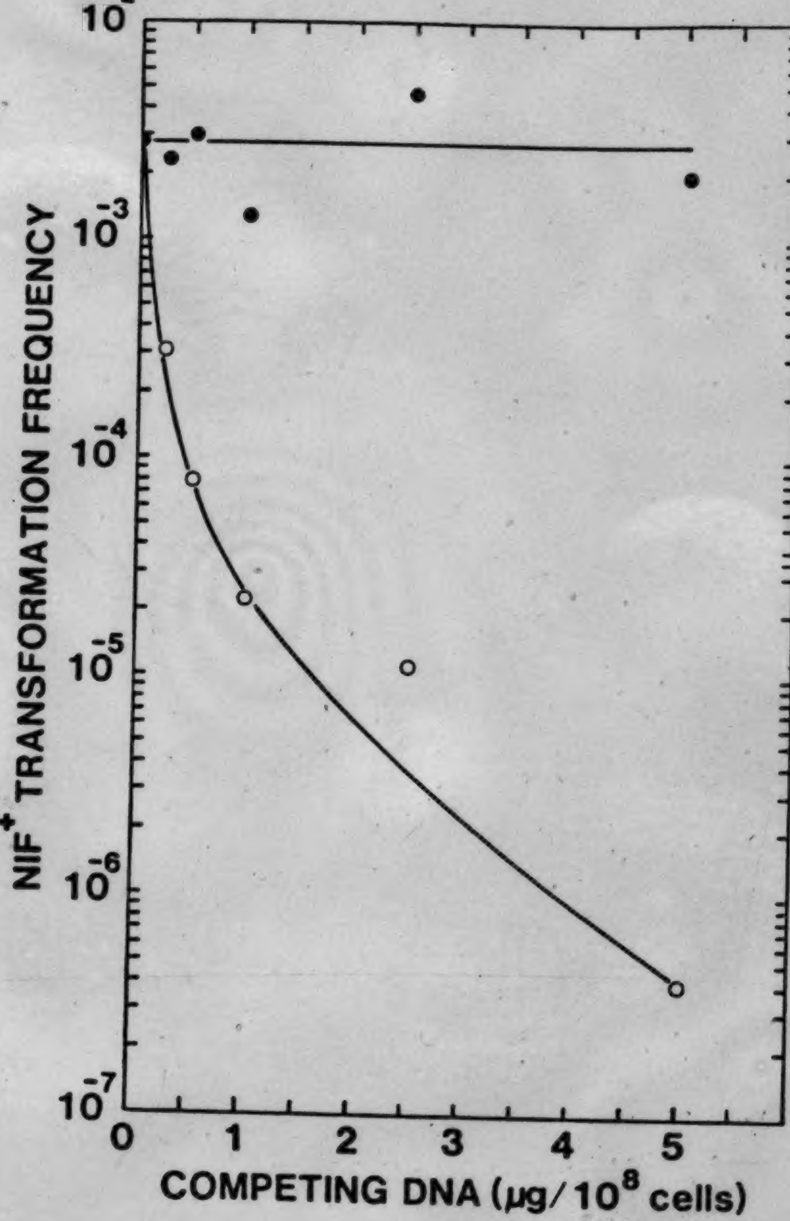
Appendix 4. Time course of derepression of nitrogenase. Srain UW was pregrown for 20 h in Fe-limited Burk medium. Cells pelleted from 5 ml aliquots of culture were washed twice by centrifugation with 2.5 ml of Fe-limited, N-free Burk medium prior to respension in 5 ml of this growth medium. The cultures were added to 10-ml Erlenmeyer flasks and incubated at 30°C. At 30 min intervals beginning immediately (zero time) nitrogenase activity was assayed as described in Materials and Methods.


NITROGENASE
SPECIFIC ACTIVITY
120
80
40
0
0
1
2
3
4
HOURS INCUBATION

Appendix 5. Examination of [^{32}P]-labelled material from cells newly transformed with [^{32}P]DNA. All radioactive material from cells transformed with [^{32}P]DNA was present in the ethanol-precipitable fraction prepared as described in Chapter IV. This material was examined following dissolution and electrophoresis in a TEA-0.5% agarose gel. DNA and RNA were visualized by ethidium bromide staining (A). Lane 1 contained λ cI857 DNA. The ethanol-precipitable material was added to lane 2. Chromosomal DNA (c) migrated as a tight band of DNA with an apparent molecular weight greater than 1.7 X 10^7. A second DNA band of chromosomal, or possibly plasmid (R. L. Slot, R. N. Reusch, and H. L. Sadoff, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H22 p.116) origin migrated at a slightly slower rate. RNA molecules (r) had been electrophoresed to the end of the gel. An autoradiograph of this gel (B) indicated that all radioactivity was present in the high molecular weight DNA fraction.

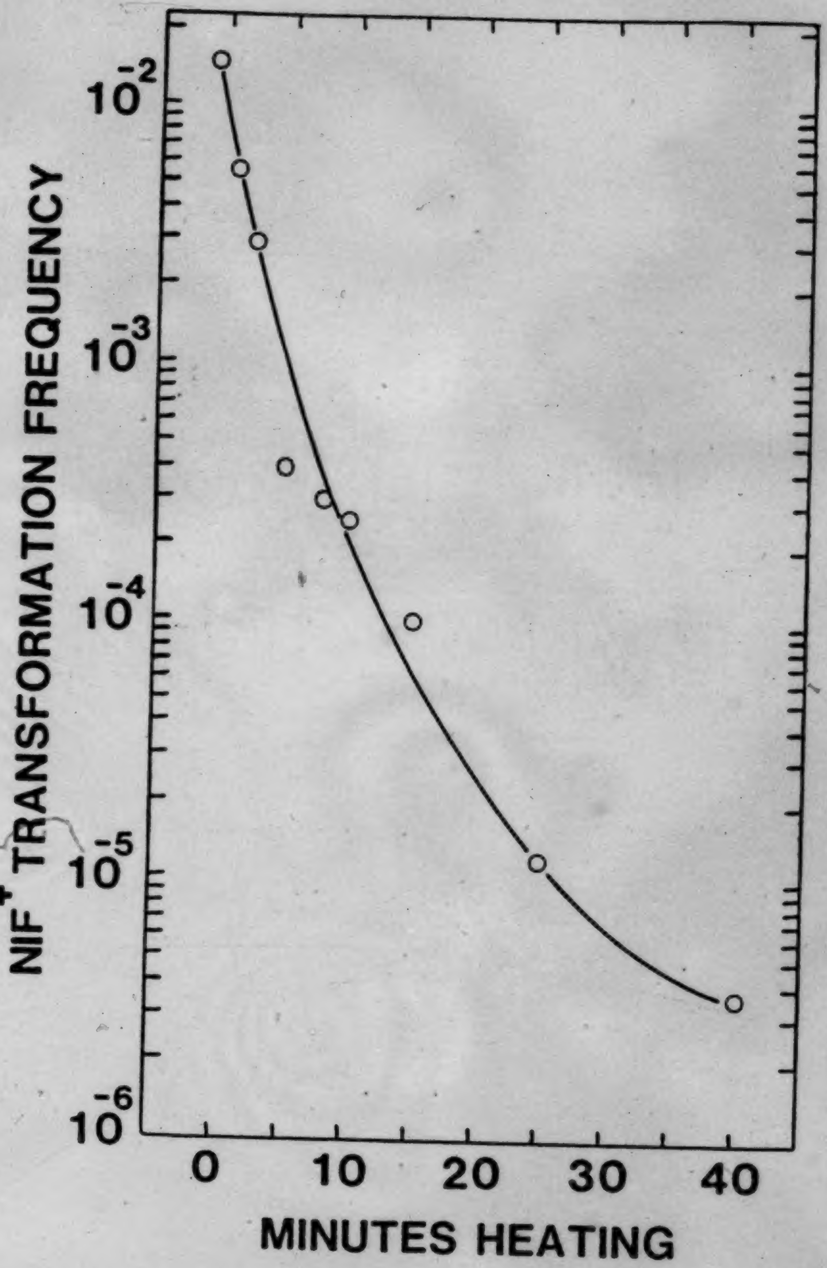

A
B
1
2
c
r

Appendix 6. Inhibitory effect of ØW-14 DNA and salmon sperm DNA on transformation of strain UW1 by homologous DNA. Competent strain UW1 cells were suspended in transformation assay buffer at 30°C and exposed to various concentrations of ØW-14 DNA (○) or salmon sperm DNA (●). Following 20 min incubation, 1.0 µg of strain 113 (Nif^{+}) DNA was added to the transformation assay. DNase-resistant DNA binding was stopped after a further 20 min incubation period by the addition of DNase 1. Nif^{+} transforma- frequency was determined as described in Materials and Methods.

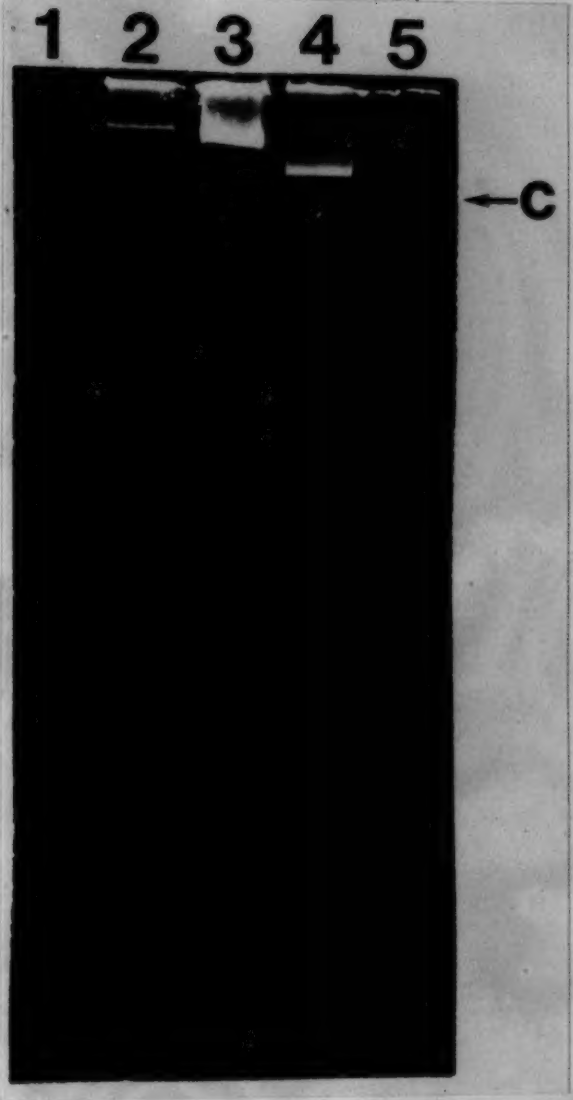


10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
NIF$^+$ TRANSFORMATION FREQUENCY
0
1
2
3
4
5
COMPETING DNA (μg/ 10^8 cells)

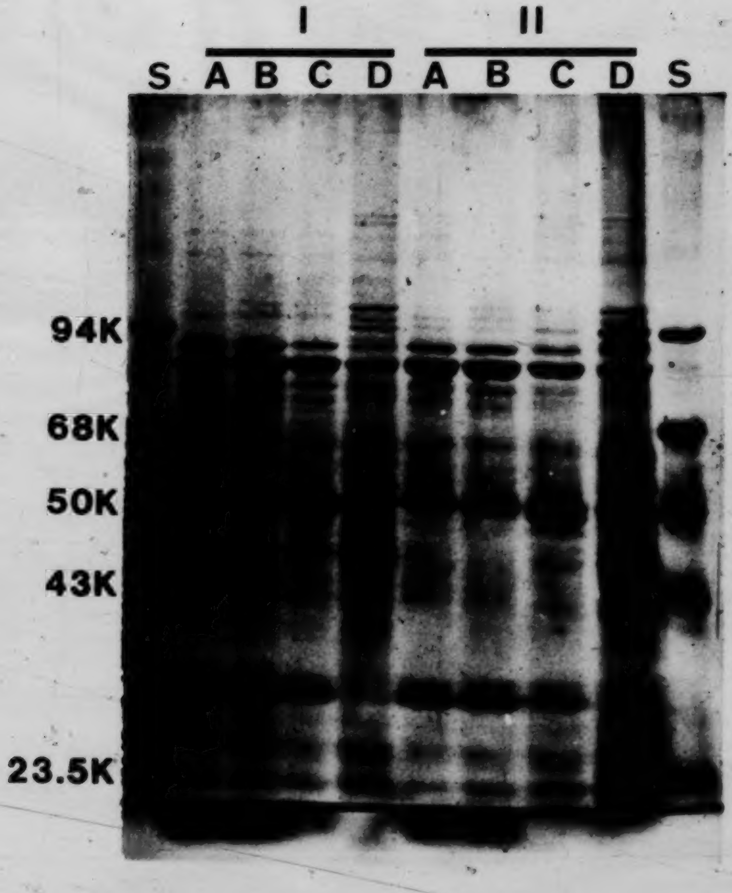
Appendix 7. Transformation of strain UW1 with heat-denatured DNA. Aliquots of crude lysate strain 113 DNA (60 μg/ml) in SSC-SDS were added to tubes preheated at 100°C. The samples were held at 100°C and at time intervals the tubes were transferred to an ice bath for 15 min. The DNA lysates were then equilibrated to 30°C and their ability to transform competent strain UW1 was determined.


NIF$^+$ TRANSFORMATION FREQUENCY
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0
10
20
30
40
MINUTES HEATING

Appendix 8. Agarose gel electrophoresis of plasmid DNA. Plasmids isolated as described in Materials and Methods were electrophoresed in TEA-0.5% agarose gels. DNA bands were detected by ethidium bromide staining and observation under UV (300 nm) light. One plasmid preparation was added to each lane of the gel. Lane 1: pDT 831 (270 kb). Lane 2: pDT 833 (180 kb). Lane 3: R1 (101 kb). Lane 4: R1-307 (54 kb). Lane 5: 1010 (8.9 kb). A small amount of contaminating *E. coli* chromosomal DNA (c) was present in some of the preparations.


1
2
3
4
5
C

Appendix 9. SDS-PAGE of proteins from 42°C-treated and unheated membrane fragments. A suspension of membrane fragments generated by French pressure disruption of competent strain UW1 was divided in half and one portion was treated at 42°C for 30 min. The unheated (I) and heated (II) membrane fragments were isolated on sucrose gradients (Page and von Tigerstrom, 1982) and the peak fractions designated (A) outer membrane peak 1, (B) outer membrane peak 2, (C) intermediate density material, and (D) inner membrane fragments were analysed by SDS-PAGE as described in Materials and Methods. Proteins were solubilized by freezing and thawing 5 times in sample buffer and samples containing 14 μg of protein were added to each well.


I
II
S
A
B
C
D
A
B
C
D
S
94K
68K
50K
43K
23.5K

APPENDIX 10. Fatty Acids from Phospholipids of Inner (IM) and Outer (OM) Membranes of Competent and Noncompetent Strain UW1.

Fatty Acid	Percent of Total Fatty Acids[a]			
	Competent Cells[b]		Noncompetent Cells[c]	
	IM	OM	IM	OM
Myristic	3.4	3.3	4.3	9.4
Palmitic	40.8	35.7	37.4	29.0
Palmitoleic	30.1	39.5	30.3	38.0
cis-9,10-methylene-hexadecanoic	0.5	0.4	0.6	0.7
Oleic	22.6	18.8	26.2	20.0
Stearic	0.7	1.1	0.3	2.1
Lactobacillic	0.7	0.8	0.7	1.2
Others[d]	0.3	-	0.1	-

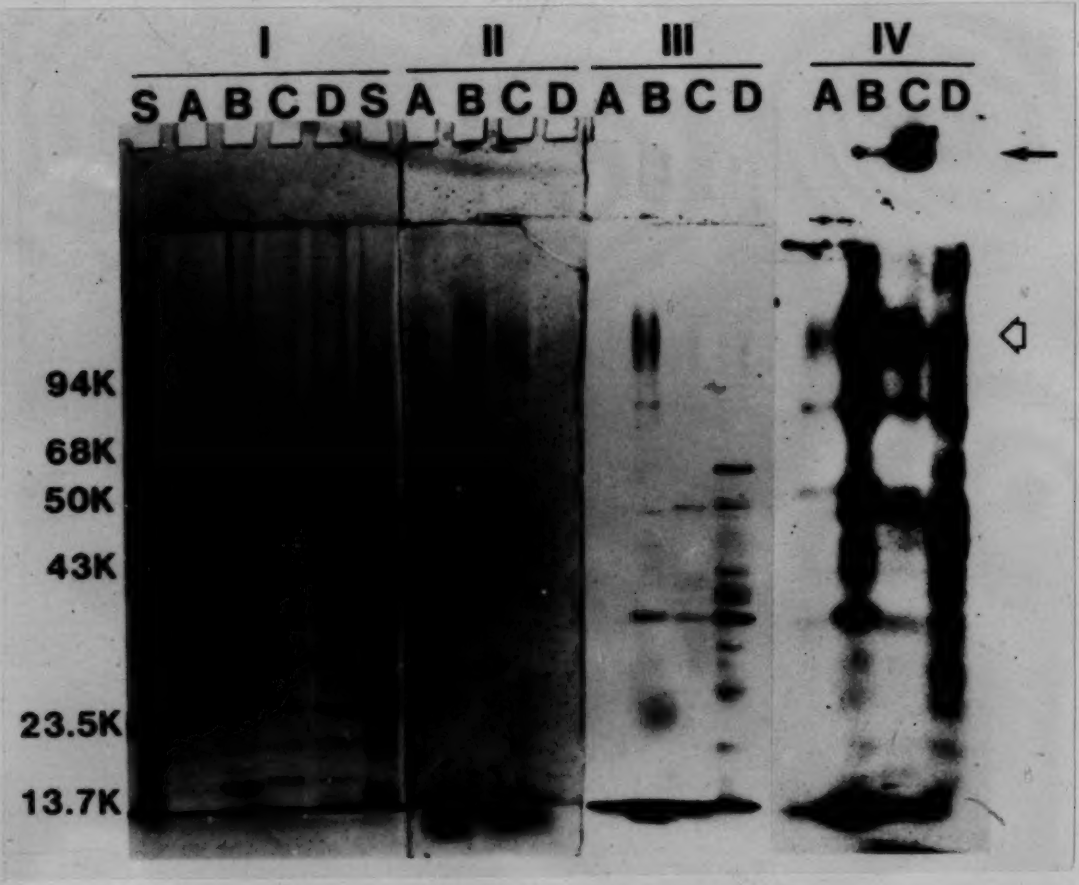
[a] Membrane vesicles were prepared as described in Materials and Methods. The percent abundance of each fatty acid methyl ester was calculated from gas-liquid chromatography data as described in Materials and Methods.

[b] Competent cells were prepared by 20-22 h growth in iron-limited Burk medium.

[c] Noncompetent cells were prepared by growth in Burk medium under otherwise identical conditions.

[d] The inner membrane preparations also appeared to contain very small amounts of pentadecanoic, methyl pentadecanoic and arachidic fatty acids.

Appendix 11. SDS-PAGE of [^{14}C]-labelled membranes. Heat-treated (42°C, 25 min), formerly-competent cells were labelled during competence recovery using [^{14}C]glucose as described in Materials and Methods. The cells were labelled for a 4 h period as cellular metabolism had become impaired by prolonged iron starvation as evidenced by the lack of increase in numbers of viable cells during 8 h post-heating incubation (Chapter IV). Cells which were highly competent prior to heating (Nif$^+$ transformation frequency 1.2 X 10^{-2}) developed low levels of competence after 8 h incubation in glucose-limited (Nif$^+$ transformation frequency 6.8 X 10^{-6}). The addition of [^{14}C]glucose to 0.2 % allowed the medium to support competence recovery during 8 h incubation (Nif$^+$ transformation frequency 1.0 X 10^{-3}) almost as well as original culture supernatant (Nif$^+$ transformation frequency 2.5 X 10^{-3}), demonstrating the need for a source of carbon for competence recovery. Membrane fragments isolated from sucrose gradients were analyzed by SDS-PAGE. Membrane material from (A) outer membrane peak 1, (B) outer membrane peak 2, (C) intermediate density fraction and (D) inner membrane fraction (see Chapter V) was solubilized by boiling for 5 min in sample buffer (Materials and Methods). The samples were applied to a single gel as four replicate sets. The molecular weight standards mixture (S) was that described in Fig. 16 with additional ribonuclease A (13,700). Following electrophoresis the gel was sliced into four sections (I-IV). After appropriate treatment of each the results were assembled for comparison. Sections I, II and III were stained with Coomassie blue R250, periodic acid-Schiff stain and a silver stain for lipopolysaccaride (some proteins were also stained), respectively. Section IV was used for gel fluorography and the autoradiograph is presented. The open arrow (▷) indicates the position of lipopolysaccaride and the solid arrow (→) indicates the migration distance of poly-β-hydroxybutyrate.


I
II
III
IV
S A B C D S
A B C D
A B C D
A B C D
94K
68K
50K
43K
23.5K
13.7K